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THE HISTOGENESIS AND CYCLIC PHENOMENA OF THE SINUS GLAND AND X-ORGAN IN CRUSTACEA

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Introduction

In the decade that has followed Hanström's (1933, 1934) description of the sinus gland and X-organ in Crustacea a number of investigators, Sjögren (1934), Hanström (1937), and Ståhl (1938) have described them in detail. All of these studies have been concerned with a description of these glands as they appear in the adult animal. Since there has been little or no work done upon the histogenesis of either the sinus gland or X-organ, it is one of the objects of this paper to describe the histogenesis of both the sinus gland and X-organ in detail.

The endocrine activity of the sinus gland has been more or less well established through numerous studies in the past several years. As these are quite adequately and critically examined by Scharrer (1941) and Kleinholz (1942) there is no need to review the literature in detail. For further information of this nature one should examine those papers. Although extensive physiological studies have been made in relation to the endocrine function of the sinus gland, there have been no cytological studies made (except in Cambarus by Dethier 1942) to determine whether or not there are any evidences of cyclic phenomena in this gland. The role of the X-organ has been suggested, but no cytological studies have been made of it. Both the X-organ and sinus gland have been cytologically examined and the results of this study are reported herein.

Methods and Materials

The histogenesis of the sinus gland and X-organ were studied in two species of Crustacea, *Homarus americanus* and *Pinnotheres maculatus*. The adults of these species and of *Cambarus virilis* were studied for cytological evidences of cyclic phenomena during the moulting period.

The eggs of Homarus were fixed in Carnoy-Lebrun: the first four stages after hatching were fixed in Zenker-formol and Bouin-Duboscq-Brasil, and the adult eye stalks (one week, 48 hours, six hours before, during, six hours, 48 hours, one,

[1] Contribution No. 326 from Woods Hole Oceanographic Institution.

one and one-half, four, six and thirteen months after moulting) [2] were fixed in Zenker, Zenker-formol and Bouin-Duboscq-Brasil. The eggs and first four stages after hatching were doubly imbedded in parlodion and paraffin and sectioned at five to nine micra. In some of the adults the exoskeleton of the eye stalk was decalcified and the whole eye stalk was doubly imbedded in parlodion and paraffin and sectioned at seven to 12 micra. In other adults the exoskeleton of the eye stalk was removed and the specimens were singly imbedded in paraffin. These were sectioned at seven to 15 micra.

The eggs, first zoea and adult stages (before, during, after and between moulting periods) of Pinnotheres were studied. The eggs and first zoea were fixed in Carnoy-Lebrun, Zenker-formol and Bouin-Duboscq-Brasil, and were doubly imbedded in parlodion and paraffin. Sections were cut at four to seven micra. The various stages of the adult were fixed in Zenker-formol and Bouin-Duboscq-Brasil, and were doubly and singly imbedded and sectioned at six to 12 micra.

The eye stalks of Cambarus were treated in the same way as those of Homarus; some were singly and some doubly imbedded. Sections were cut at seven and nine micra.

Serial sections were made of all specimens and these were stained with haemalum and eosin, Mallory's triple, Foot's (1933) and Lillie's modifications of the Masson trichrome stain, and the protargol method of Bodian (1937).

Observations

A. *Pinnotheres maculatus*

The X-organ is found in the embryo just before hatching (Figs. 1, 19) in that part of the eye which will become the median ventral side of the eye stalk in the

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Plate I [3]

The histogenesis of the sinus gland and the X-organ in *Pinnotheres maculatus*. All figures are oblique frontal sections of the right eye stalk. The neuropile of the optic ganglion is white and the ganglion cell layer is stippled.

Figure 1. Section of the late egg stage showing the position of the X-organ in relation to the structures of the optic ganglion. X—X-organ. S. G.—Sinus gland.

Figure 2. Section of the first adult stage showing the positions of the sinus gland and X-organ: both are distal to the medulla terminalis.

Figure 3. Section of the second adult stage. The sinus gland has begun to move distally, but the X-organ is found in the same general position as in earlier stages.

Figure 4. Section of the third adult stage. The sinus gland has advanced to a point between the medulla interna and medulla externa.

Figure 5. Section of the fourth adult stage. The sinus gland is lateral to the medulla externa at this stage.

Figure 6. Section of the fifth adult stage. The sinus gland now occupies a position between the medulla externa and lamina ganglionaris.

Figure 7. Section of the sixth adult stage. The sinus gland has advanced to a point that is distal to the lamina ganglionaris. In this as in previous stages the X-organ is found distal to the medulla terminalis.

[2] I am indebted to Dr. Charles J. Fish and the staff at the Wickford Hatcheries for the determination of the times in those specimens that were about to moult.

[3] All figures have been drawn with the aid of a micro-projection apparatus. All structures found between the hypodermis of the exoskeleton and the optic ganglia have been omitted for the sake of clarity.

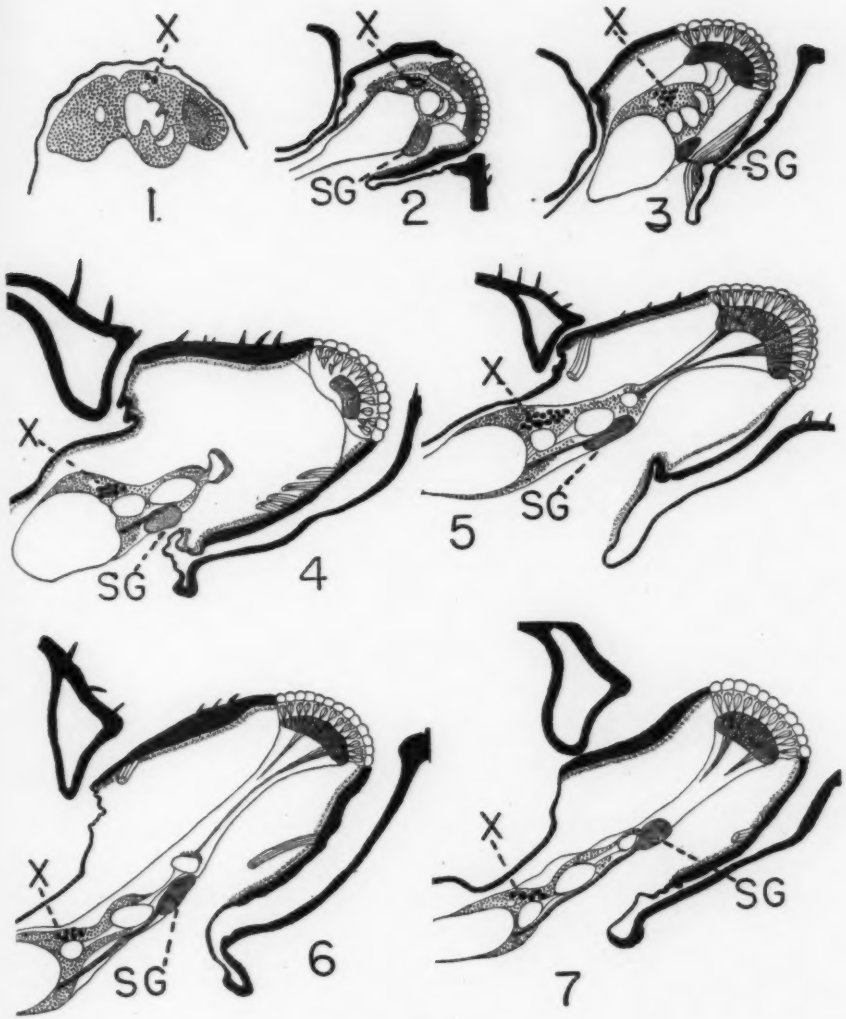


PLATE I

first zoea. It occupies a position on the distal portion of the medulla terminalis and is almost entirely surrounded by the cells of the optic ganglia. There is also found, usually between the X-organ and the medulla terminalis, an area which is devoid of cells; this appears in serial sections as a hole. As far as can be determined from cytological preparations the cells of the X-organ are very similar to those found in the ganglion cell layer. They are probably derived from the same embryonic source and later become differentiated into X-organ cells. There is no evidence of any nerve fiber connections with the medulla terminalis. The nuclei are of the same size, shape and appearance as the nuclei of the ganglia cells. There is more cytoplasm present than in the ganglia cells; it is non-granular and clear. The X-organ is an integral part of the ganglia cell layer and is not set apart from it by a connective tissue sheath.

The secretory products of the X-organ are large rounded masses which exhibit concentric rings; this seems to indicate that the secretions have been laid down at different intervals. These secretory products always give a basophilic reaction when stained; they are blue after aniline blue and are structurally very similar to those found in the X-organ of Homarus. There is no evidence of any cyclic phenomena in the egg stage as the secretions have the same characteristic appearance in all specimens.

There is no evidence that the sinus gland has been developed by the late egg stage or the first zoeal stage. Unfortunately, conditions existing at Woods Hole last summer did not permit obtaining the intermediate forms between the first zoeal and the first adult stage so that these could not be studied. Attempts to raise them beyond the first zoeal stage were fruitless.

The adults may be grouped into six categories or stages which correspond to the moults. This is comparable to the five moults found in *Pinnotheres pisum* by Atkins (1926). In all stages of the adult (Figs. 2–7, 21) the X-organ is found in the same relative position that it occupies in the egg stage. The number of cells of which it is composed increases after each moult, but no mitoses were observed at any time. The cells are larger than the ganglia cells which surround them. They are wedge-shaped and are grouped in such a way as to remind one (when examining serial sections) of a pie that has been cut; the nuclei are found around the periphery and each cell becomes narrower as its cytoplasm extends toward the center of the X-organ. As the cytoplasm becomes filled with the secretory products the nucleus is pushed more and more toward the periphery

PLATE II [4]

The histogenesis of the sinus gland and X-organ in *Homarus americanus*. All figures are oblique frontal sections; figures 8, 9, and 11 are of the left eye stalk, and figure 10 is of the right eye stalk. The neuropile of the optic ganglion is white and the ganglion cell layer is stippled.

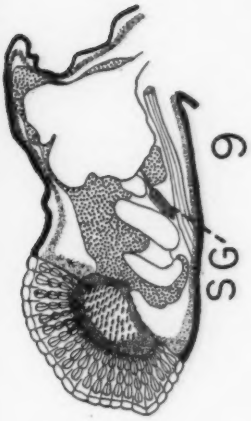
FIGURE 8. Section of the late egg stage showing the position of the X-organ.

FIGURE 9. Section of the third stage after hatching. The sinus gland is seen as a thin structure on the proximal side of the medulla externa.

FIGURE 10. Section of the fourth stage after hatching. The sinus gland still occupies a position on the proximal side of the medulla externa. The X-organ extends to the hypodermis of the exoskeleton where the eye papilla is found.

FIGURE 11. Section of the adult stage. Both the X-organ and sinus gland, on opposite sides of the eye stalk, are seen extending beyond the limits of the neurilemma which surrounds the optic ganglion.

[4] See footnote 3.



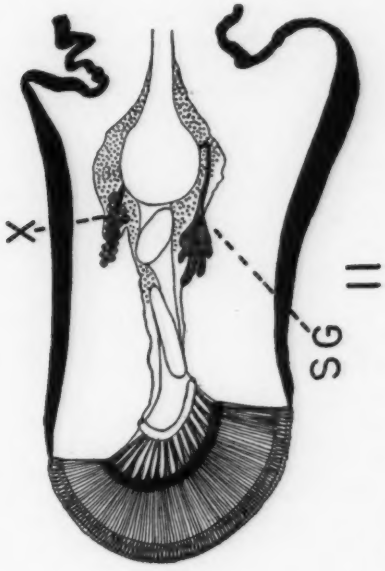
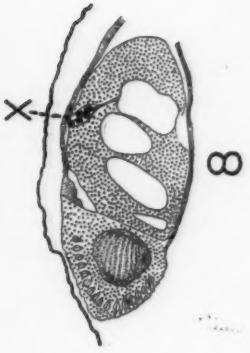
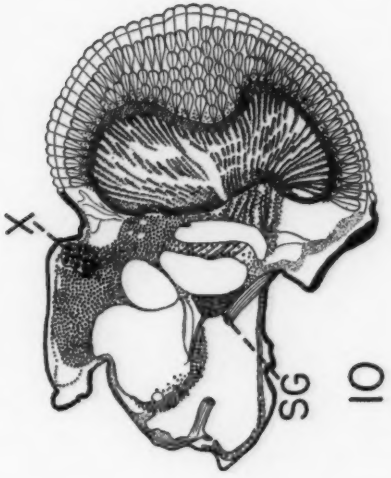


PLATE II





of the cell. In no stage is there any evidence that the X-organ has a nerve fiber tract which extends from it to the medulla terminalis. Cytologically there is no evidence of any cyclic phenomena associated with the secretion processes of the X-organ at any time. It always exhibits the same basophilic reaction regardless of whether it is fixed before, during, after or between moulting periods.

The sinus gland is well developed by the time the animal reaches the first adult stage (Fig. 2). It is at this time that it enters the mussel, *Mytilus edulis*, and begins its parasitic form of existence. The most remarkable feature in the subsequent development of the sinus gland is its change of position in relation to the structures of the optic ganglia. In the first stage (Fig. 2) it is found closely appressed to the medulla terminalis; in the second stage (Fig. 3) it has begun to move away from the medulla terminalis. In each successive stage it advances farther toward the distal portion of the eye stalk. In the third stage (Fig. 4) it occupies a position between the medulla interna and the medulla externa, in the fourth stage (Fig. 5) alongside the medulla externa, in the fifth stage (Fig. 6, 21) between the medulla externa and lamina ganglionaris, and in the sixth stage (Fig. 7) it has advanced to a point that is distal to the lamina ganglionaris.

In all stages the sinus gland is found on the dorso-lateral side of the eye stalk. There are very few nuclei in it (Fig. 23) and these bear such a close resemblance to those of the neurilemma, which is continuous with the sinus gland, that one might well consider the sinus gland a modification of the neurilemma (cf.

PLATE III[5]

Microphotographs to show the cyclic phenomena in the sinus gland of *Homarus americanus* and *Cambarus virilis*. All photographs are × 725 reduced about 35 percent, and are of materials fixed in Bouin-Duboscq-Brasil and stained with Foot's modification of the Masson trichrome stain.

FIGURE 12. *Homarus americanus*. A portion of the edge of one lobule of the sinus gland of a specimen fixed forty eight hours before moulting and sectioned at twelve micra. The brilliantly staining acidophilic secretory products are seen as dark irregularly shaped masses. This and succeeding figures show the loose network of connective tissue which constitutes the framework of the gland and the very few scattered nuclei.

FIGURE 13. *Homarus americanus*. A portion of the edge of one lobule of the sinus gland of a specimen fixed six hours after moulting and sectioned at ten micra. The bulk of the secretory masses are only slightly acidophilic and appear less dark in the photograph.

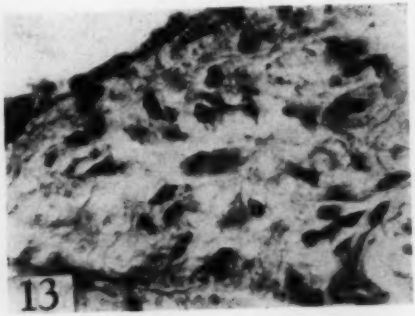
FIGURE 14. *Homarus americanus*. A portion of the edge of one lobule of the sinus gland of a specimen fixed six months after moulting and sectioned at twelve micra. The secretory material is reduced in quantity and stains in a slightly acidophilic manner.

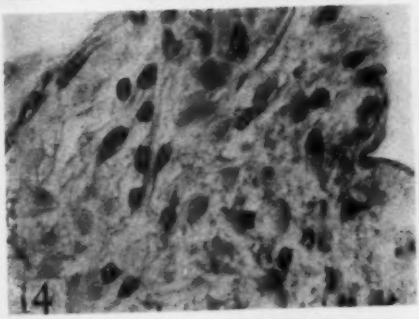
FIGURE 15. *Cambarus virilis*. A portion of the sinus gland of a specimen fixed before moulting and sectioned at nine micra. The numerous brilliantly acidophilic secretory masses are seen as dark masses hung upon the connective tissue framework of the gland. The blood sinus shows as clear areas.

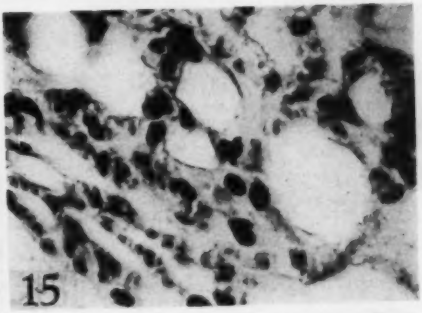
FIGURE 16. *Cambarus virilis*. A portion of the sinus gland of a specimen fixed after moulting and sectioned at nine micra. The secretory products are conspicuous by their absence. The blood sinuses are filled with blood. The nuclei are scattered at random in the loosely arranged connective tissue.

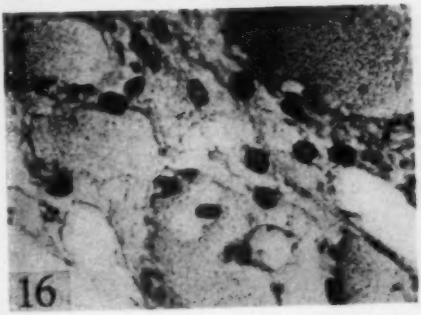
FIGURE 17. *Cambarus virilis*. A portion of the sinus gland of a specimen fixed in December and sectioned at nine micra. The majority of the secretory products present are acidophilic. The blood sinuses appear as clear areas.

[5] All microphotographs were made using Bausch and Lomb microphotographic equipment. The photographs were taken on Eastman Super Panchro Press film, and were printed on Eastman Azo F-2, and Velour Black S-4 paper. Wratten filters G No. 15 and X-1 were used.









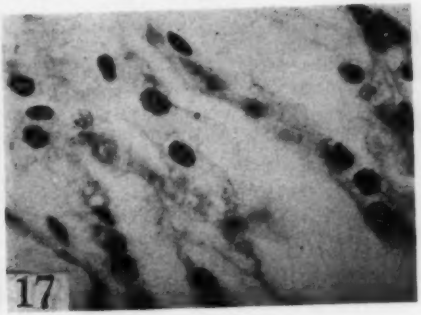


PLATE III

Hanström, 1939). In no stage of the adult is the sinus gland more than partially extruded beyond the level of the neurilemma. In all stages after the second, one finds a very large bundle of nerve fibers passing from the sinus gland toward the medulla terminalis. Some of these pass directly into the neuropile of the medulla terminalis and some ramify among the adjacent ganglion cells. In the first two adult stages the sinus gland is too closely appressed to the medulla terminalis for the presence of the nerve fibers to be readily determined. There is not, however, any evidence that there is a nerve fiber tract which extends directly to the brain as has been reported in Cambarus by Welsh (1941).

There are no obvious cell boundaries in the sinus gland. In fact there are so few nuclei to be found in any particular specimen's sinus gland that the individual cells which constitute the gland must be relatively very large. It is possible to detect canals which extend toward the blood sinus of the eye stalk; presumably these carry the secretory products to the blood stream. The secretions are in the form of large, more or less irregular, masses the amount of which varies very little regardless of the nearness or remoteness of the moulting period.

The secretions of the sinus gland give a basophilic reaction to the stains employed before, after and in the intermoult periods. In specimens fixed while in the process of moulting that portion of the sinus gland which is next to the neuropile of the adjacent optic ganglion gives an acidophilic reaction, whereas,

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PLATE IV[6]

Microphotographs to show the sinus gland and X-organ in *Homarus americanus* and *Pinnotheres maculatus*. Figures 18, 19, 20, 22, 23 × 725, and figure 21 × 150; all are reduced about 25 percent. The material shown in figure 18 was fixed in Carnoy-Lebrun, that of figure 20 was fixed in Zenker-formol, and all others were fixed in Bouin-Duboscq-Brasil. The material shown in figure 22 was stained with Mallory's triple stain, and all others were stained with Foot's modification of the Masson trichrome stain.

FIGURE 18. *Homarus americanus*. A portion of the optic ganglion of an embryo fixed in the late egg stage and sectioned at five micra. The arrow indicates the characteristic secretory products of the X-organ which is surrounded by the cell layer of the optic ganglion. (Compare with Fig. 8.)

FIGURE 19. *Pinnotheres maculatus*. Section of the late egg stage embryo showing the position (arrow) of the X-organ. Sections were cut at four micra. (Compare with Fig. 1.)

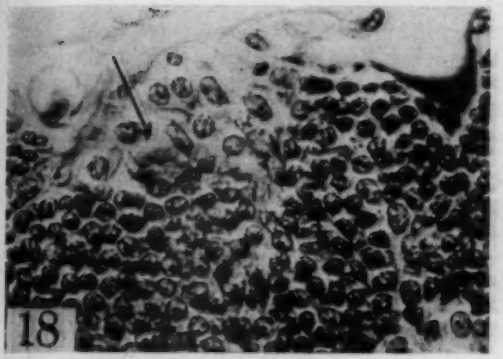
FIGURE 20. *Homarus americanus*. A portion of the eye stalk of a fourth stage embryo, sectioned at seven micra, showing the close association of the X-organ with the cells underlying the eye papilla. The bulge in the exoskeleton can be noted at the top of the photograph. Note that there are fewer nuclei in the X-organ, per unit area, than in the adjacent optic ganglion. (Compare with Fig. 10.)

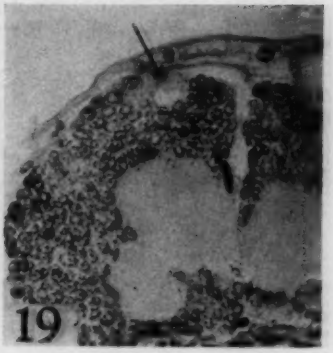
FIGURE 21. *Pinnotheres maculatus*. Section at eight micra of the eye stalk showing the general relationship of the various structures found therein. (Compare with Fig. 6.)

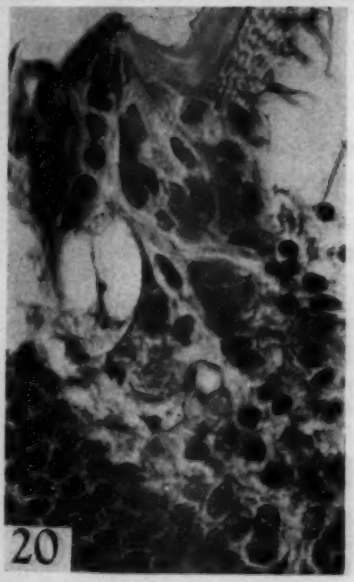
FIGURE 22. *Homarus americanus*. Section of the eye stalk of a third stage after hatching specimen (at seven micra) which shows the sinus gland lying just above the deeply staining muscle. Note that it stains much as the surrounding ganglion does and that the blood sinus is quite small.

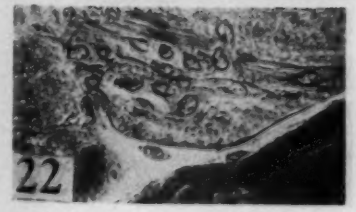
FIGURE 23. *Pinnotheres maculatus*. Section of the sinus gland shown in figure 21 enlarged to show its structure. This is the gland of a specimen that had been starved for forty-six days. Only one nucleus is to be found in this section, and what few secretory products are seen are stained brilliantly acidophilic. Note the indefiniteness to the connective tissue framework of the gland.

[6] All photographs, excepting that of figure 20, were taken using the same equipment and materials that were used for those of Plate III. The photograph for figure 20 was made on Eastman Ortho-X film using only the X-1 Wratten filter.









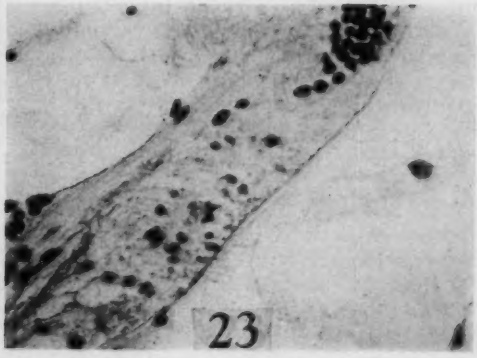


PLATE IV

the lateral portion, next to the blood sinus, gives a basophilic reaction. The acidophilic and basophilic portions blend together in the middle of the gland. Some specimens were starved for varying lengths of time. Those which had been starved for eight days exhibit both an acidophilic and basophilic reaction, but the two reactions are not regionally differentiated as is the case in specimens fixed while in the process of moulting. This reaction is found regardless of whether the specimens are fixed before, during or after the moulting period. In specimens starved for as long as 46 days (Figs. 21, 23) one finds only an acidophilic reaction regardless of the nearness or remoteness of the moulting period. Likewise, as the period of starvation is increased the amount of secretory material present in the gland is decreased although there is no evidence that the decrease due to starvation affects the frequency of moults in this particular animal.

B. *Homarus americanus*

The X-organ is found in the late egg stage (Figs. 8, 18); it is comparable in appearance to the X-organ in Pinnotheres, although there are definite structural differences in it. It is located in that part of the eye stalk that will become the median somewhat ventral side in the first stage after hatching. It is entirely surrounded by the cellular layer of the adjacent optic ganglion, but is separated from the ganglia cells by a thin connective tissue sheath. A definite bundle of nerve fibers extends from the X-organ to the medulla terminalis. The nuclei of the X-organ cells are histologically the same as those of the surrounding ganglia cells: the cytoplasm is more abundant than in the ganglia cells, and that which does not contain secretory products is clear and stains lightly. The secretory products show a series of concentric layers, when sectioned, comparable to those found in Pinnotheres; the nuclei are pushed to one side by the secretory products which nearly fill the entire cytoplasm. In all stages, under low power of the microscope, the X-organ has a similar appearance. This characteristic appearance has been described by Hanström (1939) as a "bunch of grapes." The distal portion of the X-organ extends to the median somewhat ventral portion of the hypodermis of the eye stalk. There is no evidence that there is any eye papilla formed at this time; in later stages the association of the X-organ and the eye papilla is evident. The X-organ exhibits no cyclic phenomena, cytologically, in the egg. The secretory products are always basophilic to the stains employed and vary very little in quantity.

In the first four stages after hatching (Figs. 10, 20) the X-organ increases greatly in size; this is due to the greatly increased number of cells in it and the increased amount of secretory products. Although there is a large increase in the number of cells found in the X-organ there is evidence of only an occasional mitosis after the animal has hatched. At its distal portion the X-organ comes into close contact with the exoskeleton which is bulged at this point. The cuticle of this particular region is extremely thin; this is the eye papilla (Figs. 10, 20). The eye papilla cells are found on the distal side of the X-organ between it and the ommatidia of the eye. There is no connection between the X-organ and the eye papilla as the X-organ is completely surrounded by a connective tissue sheath. No bipolar cells are found in the distal portion of the X-organ that are comparable to those described by Hanström (1937, 1939) for the adult of *Homarus*

americanus. Cytologically there is no evidence of any cyclic phenomena in the X-organ during the first four stages after hatching; the secretory products are basophilic and the quantity is quite constant.

In the adult (Fig. 11) the X-organ no longer extends to the exoskeleton, but is found in the proximal half of the eye stalk. The basal portion of it is imbedded in the cellular layer of the distal part of the medulla terminalis. The distal portion extends well beyond the ganglionic cellular layer (to a point approximately level with the distal end of the medulla interna) into the blood sinus of the eye stalk. The X-organ occupies the same general position that it occupies in the earlier stages. In general structure it has become considerably more complex; it is now divided into a large number of units each of which is composed of from ten to twenty or more cells. Each of these units has a circular, whorled appearance. The nuclei are arranged around the periphery and the secretory products occupy the central area. A large bundle of nerve fibers passes around each of the units giving off nerve fibers to the individual cells. This arrangement gives the serial sections an appearance of being a series of whorls each of which originates from a common central stem of nerve fibers. The main bundle of nerve fibers passes between the various units and extends to the median side of the medulla terminalis. The nuclei have increased prodigiously in numbers, but still bear a marked resemblance to those of the cells of the optic ganglia. The cytoplasm of the X-organ cells is large and irregular in shape; it is filled for the most part with secretory products which have the characteristic concentric layers within them. Cells not possessing secretions have a clear lightly staining cytoplasm.

There is no evidence of cyclic phenomena associated with moulting as far as the X-organ is concerned. The basophilic reaction is found regardless of whether the eye stalk has been fixed a few days, 48 hours, six hours before, six hours, 48 hours, one, one and one-half, four or six months after moulting. Likewise, there is little change in the amount of secretory products that are evident in the X-organ of the eye stalks in the above series; the number of blue staining concretions is remarkably constant. In the case of the specimen that had not moulted for more than one year there were fewer secretory products present and more of the units contained vacuoles.

As far as can be determined the sinus gland is not formed sufficiently to be definitely recognized as such until the third stage after hatching (Figs. 9, 22). At this time it is a thin, lightly staining structure located on the dorso-lateral side of the eye stalk between the medulla interna and medulla externa. It is not very conspicuous as it does not give the typically brilliant acidophilic reaction to acid fuchsin that is found in the adult sinus gland. Structurally the sinus gland has the appearance of being a thickened portion of the neurilemma which invests the optic ganglia. The nuclei are few in number and stain precisely in the same manner as the nuclei of the neurilemma. The cell boundaries cannot be discerned; the cytoplasm seems to be confined to the connective tissue framework of the gland upon which the secretory materials are hung. The general tissue of the gland, regardless of what it is composed, stains very lightly with all the stains employed. There is a definite nerve fiber tract which extends from the sinus gland to the lateral distal border of the medulla terminalis. It is this fact that makes it possible to ascertain the presence of the sinus gland in the third

stage after hatching. No such innervated structure has been found in the earlier stages.

In the fourth stage after hatching (Fig. 10) the eye stalk has increased more in thickness than in length. Consequently, the medulla interna is displaced; the sinus gland is found on the proximal portion of the medulla externa lateral to the medulla interna. This brings the sinus gland into closer proximity to the medulla terminalis. The sinus gland has increased in size with the resultant increase in the number of nuclei found in it, but the cytoplasm is still lacking the brilliant acidophilic reaction one might expect. No cell boundaries are visible; the nerve tract from the medulla terminalis is much more prominent than in the third stage after hatching.

In the adult (Fig. 11) the sinus gland occupies the same general position as in the early stages, but the eye stalk has become much more extended so that the medulla terminalis, interna and externa and lamina ganglionaris are strung out and occupy a much smaller portion of the inside of the eye stalk than they did in the early stages. As a result the sinus gland is found in the proximal half of the eye stalk on the opposite side from the X-organ. It is much more highly developed and extended than in the early stages. Situated alongside the medulla interna and extending to the proximal portions of the medulla externa it sends large finger-like processes out into the adjacent blood sinus. The nerve fiber tract extending from the sinus gland to the medulla terminalis is very large; after the protargol stain of Bodian (1937) one finds that the nerve fibers ramify among the fibers of the neuropile of the medulla terminalis and branch to all parts of the sinus gland. The framework of the gland is composed of connective tissue which stains precisely the same as the other connective tissue found in the eye stalk. There are no distinct cell boundaries observable in most preparations, but occasionally one is able to find an isolated cell which has a definite cell boundary surrounding a large irregular cytoplasmic mass. The nuclei have the same appearance as those of the early stages; they look more like connective tissue nuclei than nerve cell nuclei.

As has been pointed out above there is no striking staining reaction in the sinus gland of the third and fourth stages after hatching. In the adult, however, there are some interesting phenomena. In the series obtained for this research the following reactions are discernible: Specimens fixed several days and a few hours before moulting have the sinus gland filled with irregularily shaped secretory granules (Fig. 12) which, after Foot's modification of the Masson trichrome stain and other stains employing acid fuchsin and aniline blue, give a brilliant acidophilic reaction for the most part although there are a very few granules which react basophilically. Specimens fixed six hours, 48 hours, one and one and one-half months after moulting give three characteristic reactions. Some of the granules are brilliantly acidophilic, some are slightly acidophilic, and a number are decidedly basophilic (Fig. 13). In specimens fixed four and six months after moulting the amount of secretory material in the sinus gland is decidedly less than in those fixed during the summer months at or near the time of moulting (Fig. 14). In these cases the secretions are for the most part only slightly acidophilic with an occasional basophilic granule being found. In the specimen that had not moulted in over a year there was less secretory material in the sinus gland than was found in those (fixed in the summer months) which had moulted,

but there was more than was found in those specimens fixed in the late fall and winter. The secretory material was brilliantly acidophilic, slightly acidophilic and basophilic. There was more basophilic material in this particular specimen than in any of the others. Examination of the exoskeleton showed that a new exoskeleton had been laid down underneath the old one which had not, for some reason, been shed.

C. *Cambarus virilis*

When the cyclic phenomena were found in the sinus gland of Homarus it was thought advisable to study the sinus gland of *Cambarus virilis* in which Dethier (1942) had previously reported a similar reaction. Accordingly, sections were made of the eye stalks of specimens fixed just before and just after moulting as well as of those fixed in late December. The sinus gland of specimens fixed just before moulting (Fig. 15) was filled with many irregularly-shaped granules which for the most part gave a brilliant acidophilic reaction, but there were occasional granules which were basophilic. In those specimens fixed after having completed moulting (Fig. 16) there was a sharp reduction in the number of secretion granules present; a few of these were brilliantly acidophilic, but most of them exhibited varying degrees of a basophilic reaction. In those specimens fixed late in December (Fig. 17) there were about the same number of granules as were found in the post-moult specimens, but the majority of these were acidophilic and only a few basophilic.

Discussion

Dethier (1942) in her account of the sinus gland in Cambarus states that she has been able to trace it from the first post-embryonic moult, and that it is apparently functional at that time.[7] This is not the case in the two species used for this investigation; in Homarus it has been impossible to ascertain definitely its presence until the third stage after hatching, and in Pinnotheres it could not be detected (with the techniques used) in the egg or first zoeal stages. Cytologically the evidence seems to indicate that the sinus gland in the third and fourth stages of Homarus is not a functional gland.

It has fairly well established that the color changes in Crustacea are controlled by hormones which originate in the eye stalk. As Kleinholz (1942) points out "the glandular tissue is probably the sinus gland, although the X-organ may also be concerned in this function." The apparent absence of the sinus gland in the early stages suggests that the X-organ may be functional in this capacity at this time, but the cytological evidence does not bear this out in Homarus and Pinnotheres. On the other hand, Cambarus has no X-organ which has the characteristic concretions of secretory material that are comparable to those found in Homarus and Pinnotheres. (Welsh, 1941, has found a mass of tissue on the dorso-lateral side of the medulla terminalis which he suggests may be the X-organ in *Cambarus bartoni*.)

Megušar (1912), Abramowitz and Abramowitz (1938, 1940), Brown and Cunningham (1939), Kleinholz and Bourquin (1941), and Smith (1940) have

[7] When the crayfish hatches it is a miniature adult with all appendages etc., and is comparable to a fifth or sixth stage of *Homarus americanus*.

shown that the removal of both eye stalks from crustaceans hastens the onset of moulting. Smith showed quantitatively that the removal of both eye stalks shortened the intermoult period by slightly more than 30 per cent. This probably indicates that some structure in the eye stalk, possibly the sinus gland, produces a hormone which has an inhibiting effect upon moulting. Kyer (1942) gives good evidence that the sinus gland, when active, specifically inhibits moulting and gastrolith formation. Dethier (1942) in her account of Cambarus suggests that there is an acidophilic basophilic series which is related to the period of moulting. In the cases of Homarus and Cambarus the acidophilic reaction before moulting and the basophilic one after moulting seem to indicate cyclic changes in the sinus gland which are directly related to the moulting process. Further evidence of the activity of the sinus gland is exhibited by the reduction in the amount of secretory material in it; this is most striking in Cambarus, less evident in Homarus and scarcely detectable in Pinnotheres (this is probably due to the fact that Pinnotheres passes through several moults in fairly rapid succession).

The explanation of the basophilic and acidophilic reactions in Pinnotheres is more difficult on the basis of secretory activity. If one had only the normal animals to consider it might be possible to state that the activities of the sinus gland in this species passed through a reverse acid-base reaction which were a direct result of its activity. However, in as much as the sinus gland of the starved animals, and that in the ones in the process of moulting, both give acidophilic reactions it may be that the lack of food changes the pH- of the sinus gland from a normally basic range to an acid range. Since the animal does not feed during the period of ecdysis this may account in part for the acidophilic reaction of the sinus gland at this time.

Plankmann (1935) reported that various factors (starvation, etc.) may affect the rate of moulting. The Pinnotheres that were starved for varying periods of time were kept at a temperature comparable to that of their normal environment, on a dark background and in running sea water. There was no increase in the frequency or number of moults that occurred; it was the sinus gland that showed the affect of starvation and the X-organ appeared unchanged.

In the case of retinal pigment migration Parker (1897) could find no nerve fibers supplying the distal pigment cells in Palaemonetes. This observation started the controversy of the interrelationship of the eyes and subsequently many investigations have been made upon this subject. It has been shown in a generally satisfactory manner that the sinus gland produces a retinal pigment hormone (cf. Welsh, 1941). The question is raised as to the mechanism involved in the early stages where there is no obvious sinus gland to be found. If the sinus gland is the sole controlling factor it must be assumed that the early stages are incapable of retinal pigment migration.

Further studies are necessary to give satisfactory answers to the following points which have not been completely answered in the present study:

1. From precisely what pre-existing tissue is the sinus gland formed?
2. Is the sinus gland a syncytium?
3. Is the sinus gland noncellular and merely a storage space or are there cells which periodically fill with secretory products and break down (e.g. is secretion holocrine?)?

Summary

1. The histogenesis of the sinus gland and X-organ have been studied and described for the egg, first zoea and adult stages of *Pinnotheres maculatus*.

2. The sinus gland is not found in the egg or first zoea, but it is found in all the adult stages of Pinnotheres.

3. The X-organ is found in the egg and other stages of Pinnotheres.

4. The histogenesis of the sinus gland and X-organ have been studied and described for the egg, first four stages after hatching and the adult of *Homarus americanus*.

5. The sinus gland is not found as a definitely discernible structure in Homarus until the third stage after hatching.

6. The X-organ is found in all stages of Homarus that have been studied.

7. Evidence is presented for the existence of cyclic secretion phenomena in the sinus gland of all species studied.

8. There is no evidence of the existence of cyclic secretion phenomena in the X-organ in any of the species investigated.

Acknowledgments

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NEURULATION IN MECHANICALLY AND CHEMICALLY INHIBITED AMBLYSTOMA

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Introduction

Although the dependence of the medullary plate upon the chorda-mesoderm has attracted considerable attention from embryologists, the mechanism by which the plate becomes a neural tube has not been demonstrated.

In amphibians, it has been claimed that pressure exerted by ectoderm and mesoderm (Giersberg, 1924) or by the liquid confined between those two germ layers (Ruffini, 1925) is an active factor in neurulation. However, Lehmann (1926) and Boerema (1929), using different experimental approaches, have demonstrated that neurulation in these forms is an autonomous process within the medullary plate. In echinoderms (Moore and Burt, 1939; Moore, 1941) gastrular invagination, which in many respects resembles neurulation, has likewise been shown to be independent of ectodermal pressure.

Mitosis accompanied by a differential increase in cell volume has also been thought to be a factor in neurulation. Although little or no mitotic activity during this process was found by Glaser (1914) in *Cryptobranchus allegheniensis* or by Ruffini (1925) in Triton, the latter worker believes mitosis to be a contributing factor to neurulation in Rana. Derrick (1937) reports that the high mitotic rate in the sides of the chick medullary plate as compared with the floor may aid neurulation in that form. In this animal it has also been found that after the neural tube has closed, incidence of mitosis is higher in the evaginating optic vesicles than it is in other regions of the brain (Frank, 1925). Hutchinson (1940), on the other hand, finds that the elongation of the neural tube which occurs soon after its closure in Amblystoma is not due to cell proliferation.

The hypothesis of Glaser (1914) that neurulation in Cryptobranchus may be caused by differential water absorption in the medullary plate cells has not been supported by the data of Brown, Hamburger, and Schmitt (1941) on Amblystoma. They find no appreciable increase in the water content of the plate during the critical period as determined by density measurements. Hobson (1941), however, was able to produce unfolding of partially closed chick neural tubes by dehydrating them in hypertonic media.

Ruffini (1925) reports that neurulation is aided by autonomous, amoeboid motion of the medullary plate cells. Boerema (1929) concludes that autonomous changes in cell shape are the responsible mechanism. It is well established (Goerttler, 1925; Vogt, 1929; Manchot, 1929, and many others) that extensive

[1] This investigation was carried out under the direction of Dr. Paul Weiss. It forms a part of a thesis on "Chemical Factors in Nerve Development" presented in partial fulfillment of the requirements for the Ph.D. degree. It has been supported by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

cell movements take place within the neural ectoderm which result in the elongation of the structure, but it is not known to what extent these movements are correlated with the formation of the neural tube.

It was the purpose of the work reported here to compare the cellular changes taking place in normal embryos during neurulation with those in embryos in which neurulation had been inhibited by various means in an attempt to find some clue to the factors responsible.

Materials and Methods

Several clutches of eggs of *Amblystoma maculatum* (Shaw) and of *Amblystoma tigrinum* (Green) were used, some of which were obtained near Chicago and some of which were shipped from Pennsylvania. The eggs were reared at room temperature unless otherwise noted, and care was taken that environmental conditions should be the same for experimentals and controls in a given series. Stage numbers of all specimens refer to Harrison's tables (1918, unpublished).

Most of the embryos were fixed in modified Formol-Zenker, double embedded in celloidin and paraffin, and sectioned at 6 micra. Some specimens were stained with Ehrlich's hematoxylin and mucicarmin for the study of cell shape, nuclei, and pigment granules; others were stained with neutral gentian violet to differentiate yolk and secretion granules. A few embryos were fixed in picric alcohol and stained with Best's carmine for the determination of glycogen.

Mechanical Inhibition of Neurulation

Firstly, mechanical inhibition of neurulation was accomplished as follows: The medullary plates plus underlying mesoderm were excised from each of two *Amblystoma maculatum* embryos in Harrison's Stage 12 and explanted into Holtfreter's solution. One plate was then placed on top of the other and the two pieces of tissue weighed down with splinters of cover glass in such a manner that the plates could not fold up to form a tube. In some cases the plates were oriented so that the ectoderm of one was in contact with the mesoderm of the other; in other cases ectoderm was in contact with ectoderm. Six double explants of this type were studied. A number of intact Amblystoma eggs from the same clutch from which the membranes had been removed were reared in Holtfreter's solution, and 10 explanted medullary plates were allowed to develop freely in the same medium as controls.

The unoperated eggs developed normally except that, in some cases, the hypertonic medium caused a slight retardation of the head region. By the time the normal controls had reached Stage 28, the free explants showed distinct signs of neurulation. When the normal controls were in Stage 31 (Plate I, Fig. 1), the free explants had prominent neural folds which in some cases had nearly closed to form a tube (Plate I, Fig. 2). At the same time in the weighted explants, the medullary cells had elongated and become columnar as in early stages of normal neurulation, but the flask shape characteristic of later stages was never assumed and a tube was not formed (Plate I, Fig. 3).

There was no apparent difference in cell shape or intracellular organization between weighted explants whose ectoderm was in contact with ectoderm and those whose ectoderm was in contact with mesoderm. Thus it would seem that

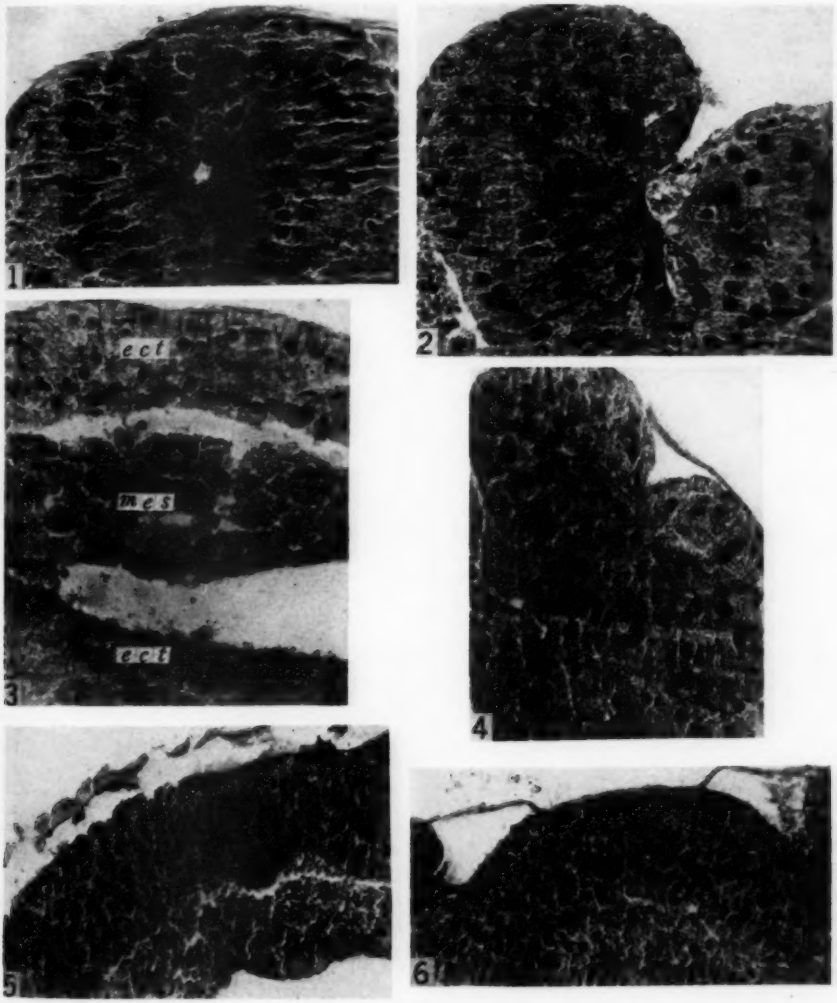


PLATE 1

FIGURE 1. Neural tube of normal *A. maculatum* embryo. 250 ×.
FIGURE 2. Medullary plate from embryo of same chronological age as Figure 1 explanted into Holtfreter's solution. 250 ×.
FIGURE 3. Double explant, same age as Figure 1. ect. = neural ectoderm. mes. = mesoderm. 250 ×.
FIGURE 4. Normal *A. tigrinum*, Stage 18. 250 ×.
FIGURE 5. Ringer-treated embryo, same chronological age as Figure 4. 160 ×.
FIGURE 6. LiCl-treated embryo, same chronological age as Figure 4. 160 ×.

by Stage 12 the dorso-ventral polarity of the neural plate has already been established. In both the weighted and free explants the cells were rounder and shorter than those in the controls, the nuclei were round as compared to the oval ones in the normal animals, and there was a heavier deposit of pigment granules around the distal edges of the medullary cells. No other significant differences were noted.

From these data it was concluded that, while pressure at right angles to the plane of the medullary plate can inhibit closure of the neural folds, it does not suppress the initial cell elongation which accompanies that closure.

Chemical Inhibition of Neurulation

Next the developing eggs were subjected to the action of lithium chloride and of hypertonic salt solutions which, in the proper concentrations, will produce delayed closing of the neural tube or permanent *spina bifida*. Three series of experiments were carried out.

Table I

Comparison of development of normal, LiCl- *and Ringer-treated Amblystoma. Figures refer to Harrison's Stages*

	Normal controls	M/10 LiCl	Mammalian Ringer's	Remarks
Series 1				
A. tigrinum	19	15	16	
20° C.	29–30	16	18–19	
	34–35	18–19	20–21	neural tube still open in head region
Series 2				
A. maculatum	16–18	13	12	
12° C.	19–20	15	12	
	22–23	18–19	—	died about 140 hours after immersion in salt solution

The first series consisted of three groups of 33 *Amblystoma tigrinum* eggs which at the inception of the experiment were in Stage 13. The first group were reared in well water to serve as normal controls. The second group were reared in M/10 LiCl solution, the third in mammalian Ringer's. A second series consisted of three groups of 17 *A. maculatum* eggs which at the beginning of the experiment were in Stage 12 b. As with the *tigrinum* eggs, one group was reared in well water, one in M/10 LiCl, and one in mammalian Ringer's. However, the *maculatum* eggs, instead of being kept at room temperature, were placed on a water table with a practically constant temperature of 12° C.

The nervous systems of the treated animals in both series diverged considerably from the mean of normal development. In general, the head region was more retarded than the spinal cord. The approximate degree of maturity attained by the experimentals in comparison with the controls is shown in Table I. In staging the treated animals, external appearance was the criterion used.

It should be noted that the difference between the normal and lithium-treated embryos is greater at 20° C. than at 12° C. (this confirms the work of Hall (1942)

on *Rana pipiens*) but that low temperatures apparently augment the effect of Ringer's solution.

The third series consisted of three groups of 17 *A. tigrinum* eggs which were in Stage 11 b—12 a at the beginning of the experiment. One group served as normal controls, one group was immersed in M/20 LiCl for 24 hours, after which development was allowed to continue in well water, and the third group was similarly treated with M/20 NaCl. NaCl treatment had no perceptible effect on the rate or type of development, while the equimolar LiCl solution retarded the embryos considerably. This series of eggs was fixed in picric alcohol for a rough determination of glycogen content.

Effects of chemical inhibition on mitotic rate

The effects of chemical inhibition were best seen in the first series of eggs as the Ringer-treated eggs did not develop at all in the second series. One-third of

TABLE II

Mitotic rate in the medullary plate of A. tigrinum. Stage numbers not in parenthesis refer to normal controls; those in parenthesis refer to inhibited animals of the same chronological age as the normal controls

	Stage	Cells counted	Mitoses seen	Mitotic index
Normal controls	18	1461	39	2.67%
	30	776	29	3.73%
	35	1037	42	4.05%
Lithium chloride-treated	18(15)	1970	19	0.96%
	30(16)	1109	12	1.08%
	35(18)	693	3	0.43%
Ringer's treated	18(16)	2276	28	1.23%
	30(18)	760	18	2.37%
	35(20)	1074	30	2.79%

the embryos were fixed and sectioned when the normal controls were in Stage 18 (at which time the normal germs had open medullary plates with well raised neural folds), one-third when the controls were in Stage 30, and the remainder when the controls were in Stage 35, by which time the lithium embryos were in approximately the same stage of development as the controls at Stage 18 as far as external appearance was concerned, and the Ringer-treated germs were slightly more mature. The effect of this inhibition on the mitotic rate in the medullary plate is summarized in Table II.

From this it is apparent first, that there is mitosis in the neural plate of *A. tigrinum* during neurulation; secondly, that the mitotic rate rises in the normal animal after the neural tube is closed; thirdly, that Ringer's solution depresses the mitotic rate in comparison with normal embryos of the same chronological age, but that the mitotic rate in Ringer-treated animals is comparable to that in normals of the same stage of development, and fourthly, that LiCl causes both a relative and an absolute decrease in the mitotic rate of the neural tube.

Effects of chemical inhibition on cell shape

The effects of inhibition on the cellular morphology of the neural tube were extreme. When the normal controls were in Stage 18 (Plate I, Fig. 4), the Ringer-treated animals showed a slight evagination of the floor of the medullary plate (Plate I, Fig. 5), and lithium-treated embryos a very marked evagination (Plate I, Fig. 6).

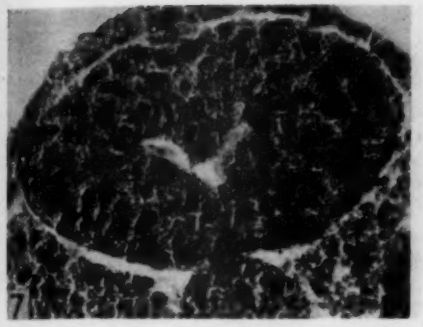
By the time the normal controls were in Stage 30 (Plate II, Fig. 7), ectoderm had begun to grow over the edges of the plate in the Ringer-treated germs and a slight invagination of the plate was present (Plate II, Fig. 8). When the controls were in Stage 35 (Plate II, Fig. 10) and the Ringer-treated embryos in what corresponded to Stage 20 in the normal animals, the invagination was fairly deep in the treated germs and the edges of the plate were raised, although they were not bent over as normal neural folds are at that time (Plate II, Fig. 11).

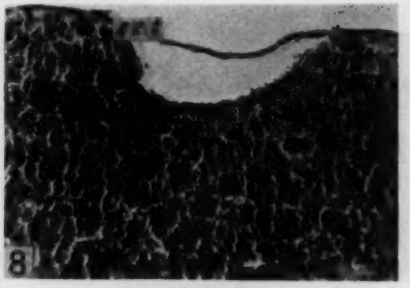
In the LiCl-treated germs, on the other hand, when the controls were in Stage 30, a flat plate was present (Plate II, Fig. 9). When the controls had reached Stage 35 and the lithium-treated animals were in Stage 18 as far as external appearance was concerned, the neural plate was still flat, but a few flask-shaped cells had appeared at the edges as in the first stages of normal neurulation. Many of the medullary plate cells in these embryos, particularly in the head region, became round and sloughed off into the space above the plate (Plate II, Fig. 12). Child (1941) reports a similar dissociation of the endodermal plate in the starfish, Pateria, when exposed to the action of lithium chloride.

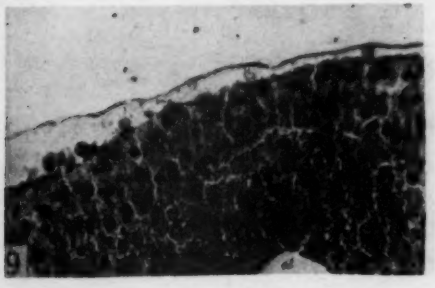
The changes in shape occurring in both the normal and treated embryos naturally correspond to the changes in the shape of the plate as a whole. These changes may be summarized by saying that both Ringer and LiCl treatment produce, first, a more or less evaginated medullary plate and then a flat or slightly invaginated plate which may, according to the concentration of the chemicals used, proceed to form a tube in places or to be overgrown by ectoderm, and that no traces of a neural keel as described by Baker (1927) were seen in the treated embryos in the series studied.

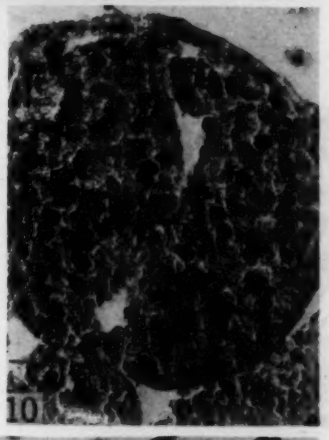
Effects of chemical inhibition on nuclear size

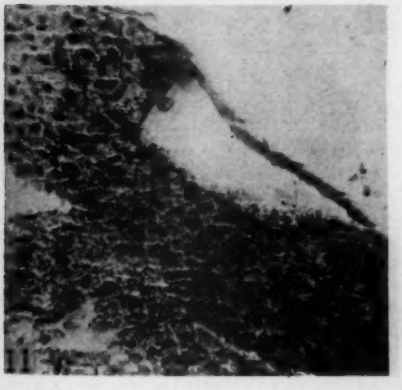
Much importance has been attached to changes in cell and nuclear size during neurulation since Glaser (1914) found that, in Cryptobranchus, the volume of the neural plate increased during the course of neurulation and believed that this indicated an increasing water content of the neural plate. He also inferred that increased hydration occurs during gastrulation in echinoderms because of reported increases in nuclear size during that process. As Brown, Hamburger, and Schmitt (1941) found no indications of increased hydration in density measurements on Amblystoma, an effort was made to throw more light on the problem by measuring the nuclear axes of 100 medullary plate cells in both normals and experimentals in each of three stages. As these nuclei are not perfect spheres and as their orientation varies somewhat within the plate, these measurements cannot be used to calculate nuclear volume. However, any large changes in nuclear volume should be revealed by this method. Indices of nuclear area and shape were also calculated. These data are summarized in Table III.











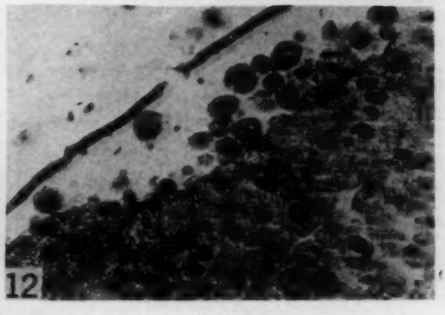


PLATE II

FIGURE 7. Normal *A. tigrinum* embryo, Stage 30. 250 ×.

FIGURE 8. Ringer-treated embryo, same chronological age as Figure 7. ect. = ectoderm growing over medullary plate. 160 ×.

FIGURE 9. LiCl-treated embryo, same chronological age as Figure 7. 160 ×.

FIGURE 10. Normal *A. tigrinum* embryo, Stage 35. 250 ×.

FIGURE 11. Ringer-treated embryo, same chronological age as Figure 10. 160 ×.

FIGURE 12. LiCl-treated embryo, same chronological age as Figure 10. Note round cells sloughed off from plate. 250 ×.

No statistically significant differences in nuclear axes, area, or shape were revealed by this analysis between normal and treated nuclei because of the large standard deviations involved. However, it should be noted that the index of shape (A/B) increased consistently in the normal germs whereas it remained practically constant or decreased slightly in the chemically treated germs. This lack of nuclear elongation seems to be correlated with the failure of cell elongation which was also observed in these cases. While no conclusions can be drawn from these findings as to cellular hydration, there is no change of nuclear size during folding of the neural plate.

Effects of chemical inhibition on cellular inclusions

A. *Yolk granules.* In normal *A. tigrinum* and *A. maculatum* embryos, yolk begins to be utilized in the neural tube, beginning in the head region, about

TABLE III

Greatest nuclear length (A) *and diameter* (B) *of 100 medullary plate cells. Stage numbers not in parenthesis refer to normal controls; those in parenthesis refer to treated animals. All measurements are in ocular micrometer units*

	Stage	Nuclear measurements				Indices			
		Length (A)		Diameter (B)		Area (AB)		Shape (A/B)	
		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Normal controls	18	15.00	2.22	8.84	1.58	129.4	34.8	1.72	0.35
	30	14.60	2.76	8.42	1.56	119.2	30.6	1.80	0.51
	35	16.80	2.83	8.86	1.52	146.6	36.8	1.93	0.48
Ringer-treated	18(16)	13.55	2.41	8.66	1.56	115.9	33.4	1.59	0.38
	30(18)	14.33	2.24	9.03	1.71	126.7	38.8	1.58	0.36
	35(20)	13.27	2.18	9.42	1.56	121.7	32.2	1.42	0.32
LiCl-treated	18(15)	14.79	2.21	9.51	1.46	139.7	34.4	1.45	0.27
	30(16)	14.63	2.22	10.79	1.56	157.3	38.0	1.26	0.25
	35(18)	13.96	2.03	10.02	1.17	138.7	32.0	1.38	0.20

Stage 18. By Stage 35, yolk has practically disappeared from the brain, and only a sparse scattering of granules remains around the lumen of the spinal cord. Bragg (1939), who has studied the utilization of yolk in a number of other amphibian genera, reports that in his animals it did not begin until after the closure of the neural tube.

Lithium chloride and Ringer's solution both seem to retard the disappearance of yolk as well as the closure of the neural tube, as the medullary plates of the treated animals were packed with yolk granules throughout the period of observation whereas, in the normal controls, the amount significantly diminished. It is doubtful if this is causally related to the process of neurulation, however, because (1) yolk disappears very late in this process and (2), as Morgan (1906) has shown, eggs of *Bufo variabilis* centrifuged so that all granules are thrown out of portions of the head in the resultant embryos will develop closed neural tubes.

B. *Glycogen.* In the *A. tigrinum* series, no perceptible change in the glycogen content of the nervous system was noted between Stage 18, at which time the neural tube is open over its full length, and Stage 30, when the entire tube is closed and morphogenesis of the brain is well under way. All the neural cells contained much glycogen, no significant differences being noted among the various regions of the nervous system.

Treatment with M/20 NaCl did not affect glycogen distribution (as was to be expected since no morphological changes were observed) nor did treatment with M/20 LiCl. Thus, although no histological method is exact enough to reveal very small changes in glycogen content, it would appear that in *A. tigrinum* neurulation is not accompanied by significant utilization of this material.

C. *Pigment granules.* Early in the normal process of neurulation in Amblystoma, as reported by Ruffini (1925) and Lehmann (1926) for other urodeles, there is a marked accumulation of pigment, especially in creases formed by the medullary folds. From the pigment layer at the distal ends of the cells, rows of pigment granules extend along the cell boundaries (see Plate I, Fig. 1). When neurulation is completed, there is a layer of pigment granules along both surfaces of the neural tube and many granules along the cell boundaries and within the cell bodies.

The chief difference noted in the treated animals was that the concentration of pigment near the outer surface of the plate cells occurred irregularly and only in those cells where shrinkage of the inner surface took place (see Plate I, Figs. 5 and 6; Plate II, Figs. 8 and 12). In the lithium-treated specimens, as soon as degeneration of the plate commenced, many granules escaped into the free space above the plate, and all of the sloughed-off cells were packed with pigment. These granules, like yolk, however, appear to be a passive factor in neurulation and are of value only as an indicator of the results of active processes which change cell shape.

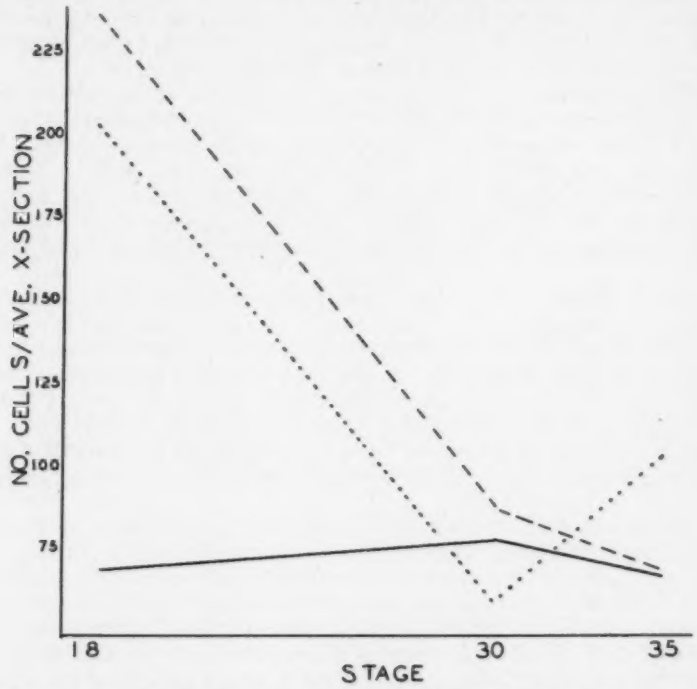
D. *Secretion granules.* As shown by Studnička (1900) and Weiss (1934), secretion occurs in the embryonic ependyma. On the chance that secretion might be involved in the process of neurulation, normal and chemically inhibited *A. tigrinum*, *A. maculatum*, and *Rana pipiens* germs and normal chick embryos which had been stained with neutral gentian violet were examined for secretion granules. None were found, either in the normal embryos or in the treated amphibians. This does not necessarily indicate that unformed secretion does not occur; in fact, the presence of liquid within the lumen of the neural tube is evidence that secretion of some sort does take place very early in normal development. Because of the difficulty of demonstrating secretion antecedents, however, the problem requires study by more refined techniques.

Effects of chemical treatment on cellular movements

The most accurate method of following cell movements in embryonic development is by vital staining, a procedure not used in this investigation. However, some indication of those movements was obtained by counting the number of cells in the neural plate in every fifth section of the trunk region of embryos (exclusive of brain and tail) and averaging the results. It is very hard to obtain strictly comparable results by this method because, as Manchot (1929) has shown, in the normal development of urodele embryos, the anterior two-thirds of the neural

plate becomes brain while the posterior one-third elongates to form the spinal cord. A rough comparison between the normal and chemically treated neural plates at various stages of development is presented in Graph 1.

As this graph was constructed from data on only nine animals, not too much significance can be attached to it. However, it would seem that in normal animals between Stages 18 and 35 mitosis and stretching of the neural plate keep pace with one another so that the average number of cells per cross section remains



GRAPH 1. Average numbers of cells present in every fifth section of the medullary plate of normal and chemically treated *A. tigrinum* embryos of the same chronological age. Abscissae are stage numbers of normal controls. Solid line used for normal controls, dashes for lithium embryos, dots for Ringer germs.

approximately constant and that, although LiCl and Ringer's solution do inhibit the stretching process just as they inhibit neurulation and mitosis, elongation of the neural plate continues under their influence.[2]

[2] In Glaser's work on Cryptobranchus (1914), he used the average number of nuclei present per cross-section to test whether or not cell division was occurring. Because the number of nuclei remained approximately constant, as it does in normal Amblystoma during slightly later stages, he concluded that there was little or no mitosis during neurulation. It would be interesting to re-examine his material to see if elongation of the medullary plate played any role in that constancy.

Discussion

In discussing neurulation, it must be borne in mind that the folding of the neural plate is a very complex process involving not only changes in cell size and proportions, but physical and biochemical changes which have, as yet, been little studied. From the foregoing analysis, it can be concluded that certain factors are not involved in the more obvious phases of folding. Thus not only does neurulation occur, as has been previously reported by many workers, in the absence of normal mechanical pressures, but the characteristic preliminary cell elongation takes place when pressure is exerted at right angles to the usual direction of cell movement.

Neurulation seems to be independent of nuclear area, and if, as has been suggested, the latter be accepted as an index of cell hydration, also independent of hydration. For, although treatment with Ringer's solution and LiCl had no significant effect on nuclear area, it did inhibit folding of the medullary plate. On the other hand, nuclear elongation, which accompanies cell elongation in the normal plate, does not occur when folding is inhibited.

Judging by the data on average number of cells per section, the elongation of the medullary plate which normally takes place during and after neurulation is not necessarily correlated with the closure of the neural tube, because, although both LiCl and Ringer's solution do retard the stretching process somewhat, it continues even when neural folds do not form.

Mitosis seems to be either directly instrumental in neurulation or at least under control of the mechanism of neurulation. Thus in the LiCl-treated embryos, in which the mitotic rate fell to a very low value during the experiment, no folds appeared, while the elevation of the sides of the neural plate in both the normal and the Ringer-treated specimens was accompanied by active cell division.

Cell elongation and wedging are unavoidably correlated with embryonic folding, and, as Boerema (1929) and others have pointed out, such changes are theoretically quite sufficient to cause neurulation. As demonstrated by Brown, Hamburger, and Schmitt (1941) differential water absorption cannot account for such changes in Amblystoma. These workers and Schmitt (1941) independently have suggested that molecular interactions and desolvations in the cell surface may exert the forces necessary to cause cell elongation. Weiss (unpublished) has suggested further that the concentration of pigment granules which occurs in the normal folding plate indicates a contraction of the cell cortex at the free surface. Although Hobson (1941) has not succeeded in demonstrating any systematic changes in the ultrastructure of the chick neural plate during folding by polariscopic analysis, a more intensive investigation of such changes during neurulation appears to be the most promising method of approach to the problem.

An interesting point which emerges in a comparison of the LiCl and Ringer-treated germs is that the effects of the two agents on neurulation seem to be produced by different means. Thus lithium is less effective at low temperatures, while hypertonic salt solutions are more effective. Further, lithium salts inhibit neurulation at much lower concentrations than do those present in Ringer's solution. Hall (1942) has evidence that lithium is a toxic agent acting on the chordamesoderm rather than on the responding ectoderm. The fact that Ringer's solution is a more effective inhibitor at low than at high temperatures would suggest that it acts on the physical consistency of the embryo rather than on chemical

processes, perhaps by stiffening the neural plate so that folding is impeded—a suggestion which Giersberg (1924) has previously offered to explain the action of sucrose and sodium acetate on neurulation.

Finally, it should be noted that the data presented here are by no means conclusive in themselves; they are offered merely in an effort to shed light on a few phases of a very complicated problem.

Summary

1. Mechanical pressure exerted at right angles to the plane of explanted medullary plates has been found to suppress neurulation in *Amblystoma maculatum*, but not the preliminary cellular elongation which is normally involved in that process. This elongation takes place irrespective of whether the medullary plate is in contact with ectoderm or with mesoderm on the normally free surface.

2. In *Amblystoma tigrinum* and *A. maculatum* neurulation is accompanied by mitosis, the mitotic rate rising after the neural tube has closed. Treatment with mammalian Ringer's solution at room temperature decreases the mitotic rate to about the same degree as it inhibits normal development; treatment with M/10 LiCl decreases the mitotic rate both relatively and absolutely.

3. No statistically significant difference was found in average nuclear area between normal and treated medullary plates. In normal germs, the nuclei elongate during neurulation, whereas in the treated germs they did not.

4. Glycogen and yolk begin to disappear from the normal neural tube about Stage 18. Neurulation-inhibiting chemicals retard the utilization of these substances.

5. Pigment granules appear to be passive factors in neurulation indicative of contraction at free cell surfaces.

6. No evidence of formed secretion from the neural plate was found.

7. Although inhibiting chemicals decrease the rate of elongation of the medullary plate, stretching continues even when neural folds fail to form.

8. The inhibiting action of LiCl is less effective at low temperatures, that of Ringer's is augmented.

9. It is concluded that neurulation in Amblystoma is autonomous to the medullary plate and may be aided by mitotic activity; changes in nuclear area (which may be indicative of cell hydration), intracellular inclusions, and longitudinal cell movements are not instrumental in the process.

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ANALYSIS OF POPULATION DEVELOPMENT IN DAPHNIA AT DIFFERENT TEMPERATURES

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Introduction

The purpose of this study was to analyze the development of Daphnia populations under controlled conditions in which temperature was the chief variant. It was proposed to investigate not only the effect of temperature upon the rate of increase, but also its influence upon subsequent changes in the numerical strength of the population. The original intention was to measure the effect of temperature by comparing the equilibrium values, i.e. asymptotes, attained by populations at different temperatures, and through further experiments to identify the processes by which temperature might act to bring about the observed differences. However, the type of population growth curve obtained precludes the comparison of asymptotes and necessitates a brief historical sketch of population studies in general and the curves developed from them, in addition to a review of previous research upon the effects of temperature.

Since the animal chosen for the investigation is a planktonic form, the analysis of the relation of temperature to the development of a population acquires additional interest from an old controversy. It has long been held that the polar regions support a more abundant, if a less diversified, fauna and flora than do the tropics. This contention has been stressed especially in connection with the latitudinal distribution of plankton, and a number of theories have been advanced relating temperature, directly or indirectly, to the density of planktonic populations. The relation of the present study to this problem and other possible applications will be dealt with in the discussion.

Historical Background

The logistic equation and its sigmoid curve, rediscovered by Pearl and Reed (1920), have been applied to the study of human populations (Pearl, 1925) and experimental populations of a variety of organisms, including yeast cells (Clark, 1922; Richards, 1928), diatoms (Ketchum and Redfield, 1938), infusorians (Robertson, 1921, 1923), and flour beetles (Chapman, 1928; Holdaway, 1932). Indeed no population study under controlled environmental conditions has demonstrated any other type of population growth curve. It should be noted, however, that the interest of investigators of experimental population development has been focussed almost exclusively on the early parts of the growth curve, with very little regard for the important part of the history which follows the initial period of increase. Although the definition of a logistic curve requires an upper asymptote, some workers have followed the development of their experimental populations only to the point where they first approach an apparent maximum size, and in

presenting their results have termed their curves logistic. While it is true that in several studies (e.g. Pearl with Drosophila, 1925; Gause with yeast, 1932; Chapman with Tribolium, 1928) the population has maintained an upper asymptote for a period long compared to the "growth" period, it does not follow that populations of other species or under different circumstances would yield similar results. The tacit assumption that an asymptote can be calculated from the maximum size reached by a population, without experimental evidence that a state of relative equilibrium has been attained, is entirely gratuitous. It may well be that the failure to demonstrate, hitherto, a type of population growth curve that is not logistic after the initial period of increase, has been due in part to this fallacious assumption.

The rather extensive literature on population studies yields but a meager amount of information concerning the effects of temperature. The yeast *Saccharomyces cerevisiae* has been the subject of two investigations involving temperature. Richards (1928a) found that the rate of multiplication increases with temperature between 4° and 30° C.; above this range it decreases. In a more thorough analysis of the effects of temperature, Gause (1932) followed the development of yeast populations to their asymptotes and discovered that in a temperature range of 5.7° to 41.0° C. the relation between the size of the asymptotic population and the temperature can be expressed by a bell-shaped curve with the mode at about 24° C. In the same paper Gause reported that populations of Drosophila held at 29° C. attain an asymptote of 310, whereas at 30° the asymptote is only 146.

Terao and Tanaka (1928, 1928a, 1928b, 1930) attempted the study of the influence of temperature on population development in *Moina macrocopa*, but followed their population growth curves only to apparent maxima, and based their conclusions on the calculated values of undemonstrated asymptotes.

Materials and Methods

Daphnia magna would appear to be ideal material for population studies because of its size, high reproductive capacity, and parthenogenesis, which makes it easy to obtain genetically identical material. Daphnia is less easily provoked to the production of males and sexual females than other cladoceran genera (e.g. Moina), so that in a crowded population a very high percentage of the individuals are potential producers, and there is no problem of a proper balance of sexes. In these experiments the sex ratio was noted at irregular intervals, and at no time did the males constitute more than 10 per cent of the population. Since cladocerans pass their early stages in the maternal brood pouch whence they are released in active state, there are no stages (such as eggs) so small as to require special precautions against loss during transfer of the population to fresh medium.

The populations developed in 50 cc. of filtered pond water from the Middlesex Fells, in open, widemouthed glass bottles whose water-surface area was 10.9 cm.^{2} The seeding of each bottle was two animals (parthenogenetic females) that had been released from the brood pouch within 24 hours. Each population was counted every two days, at which time the dead were removed, their number noted, and the water changed. This was done by pouring the contents of each bottle into a fingerbowl, whence the animals were transferred with a pipette to a

second fingerbowl containing about 50 cc. of fresh pond water at the same temperature, and thence into a clean bottle which was finally filled up to the 50 cc. mark with fresh pond water. By this rinsing process the small amount of used water carried over in the pipette was greatly diluted. Thus "conditioning" of the medium by the metabolic activities of the animals was never allowed to proceed for more than two days.

The only food used was *Chlorella pyrenoidosa*, a unicellular green alga that will grow in a thick suspension when properly cultured (in Detmer's Solution, exposed to neon light, with carbon dioxide bubbling through the medium). It was found necessary to culture the Chlorella under sterile conditions to prevent the development of a concentrated bacterial flora in the culture flasks. In previous experiments contamination of the Daphnia medium from this source had occasionally been sufficiently severe to injure the animals. The quantity of food given each population was not measured by any absolute standard. In each case it was roughly calculated, by previous experience alone, to exceed the requirements of the particular population. This method proved entirely satisfactory, for the medium always had a distinct greenish tinge. It was also demonstrated by simple experiments that when the concentration of the Chlorella was half as great or several times as great as the concentration that normally would have been used under the given conditions, the longevity and reproductive rate of the animals were not appreciably affected. Therefore neither a lack nor an excess of food was ever a limiting factor in the growth of the populations.

The temperatures chosen for comparison were 12°, 18° and 25° C., covering a considerable portion of the range (8° to 28° C.) demonstrated suitable for the life and reproduction of *Daphnia magna* (MacArthur and Baillie, 1929). At 12°, however, populations persisted for only a few weeks of faltering growth and rapidly dwindled to extinction. Under the ecological conditions that obtained, apparently the metabolic rate was not high enough to insure the reproductive and survival rates requisite for population growth and maintenance. In consequence the lowest temperature was abandoned and the work was limited to two temperatures, 18° and 25° C. The populations were maintained at these temperatures (plus or minus 1° C.) by keeping them in incubators in a cold-room.

The culture bottles were placed in daylight from a north window. However, all the populations received approximately the same amount of light. Aside from this, no attempt was made to control light conditions, which varied from day to day and from season to season.

The only environmental agencies that suggest themselves as possible limiting factors in the growth of populations of such an animal as Daphnia are: 1. exhaustion of the food supply and 2. conditioning of the medium by the accumulation of metabolites and/or depletion of the dissolved oxygen. Since the former was never operative in these experiments, any limitation in the increase in numbers must have been the expression of some form of conditioning of the medium, although that process was never continuously sustained for more than two days. In an attempt to ascertain the nature of this conditioning, the concentrations of hydrogen ion, dissolved oxygen and free carbon dioxide in the culture medium were determined at various densities of population.

The pH, as determined with a Hellige Comparator, never left the range 6.9–7.1, and even within these narrow limits it was not correlated with the popula-

tion density nor with the length of time that animals had been living in the water. It can be said with a fair degree of certainty that the hydrogen ion concentration never exerted an important influence upon population growth.

The concentration of free carbon dioxide was determined by a titration method reported in a publication of the American Public Health Association (1936). Thus determined, the amount in unused pond water at 18° or at 25° was imperceptible. At the end of a two-day period, crowded populations at 18° had raised the concentration to an average of 4.97 p.p.m.; at 25°, to 5.66 p.p.m.

Winkler Method determinations of the dissolved oxygen concentrations of fresh pond water and water conditioned by large populations for two days yielded the following results: 1. At 18°, unused water 8.38 p.p.m.; after two days' conditioning 6.59 p.p.m. and 2. at 25°, unused water 7.57 p.p.m.; after two days conditioning 4.81 p.p.m. Each of these figures is the average of ten determinations.

There is no evidence available at the present time as to whether or not these slight changes in carbon dioxide and oxygen are sufficient to account for the limitation in population growth. It is also possible that the limiting factor be some metabolite such as that postulated by Brown and Banta (1932) for male production.

Course and Analysis of Population Development

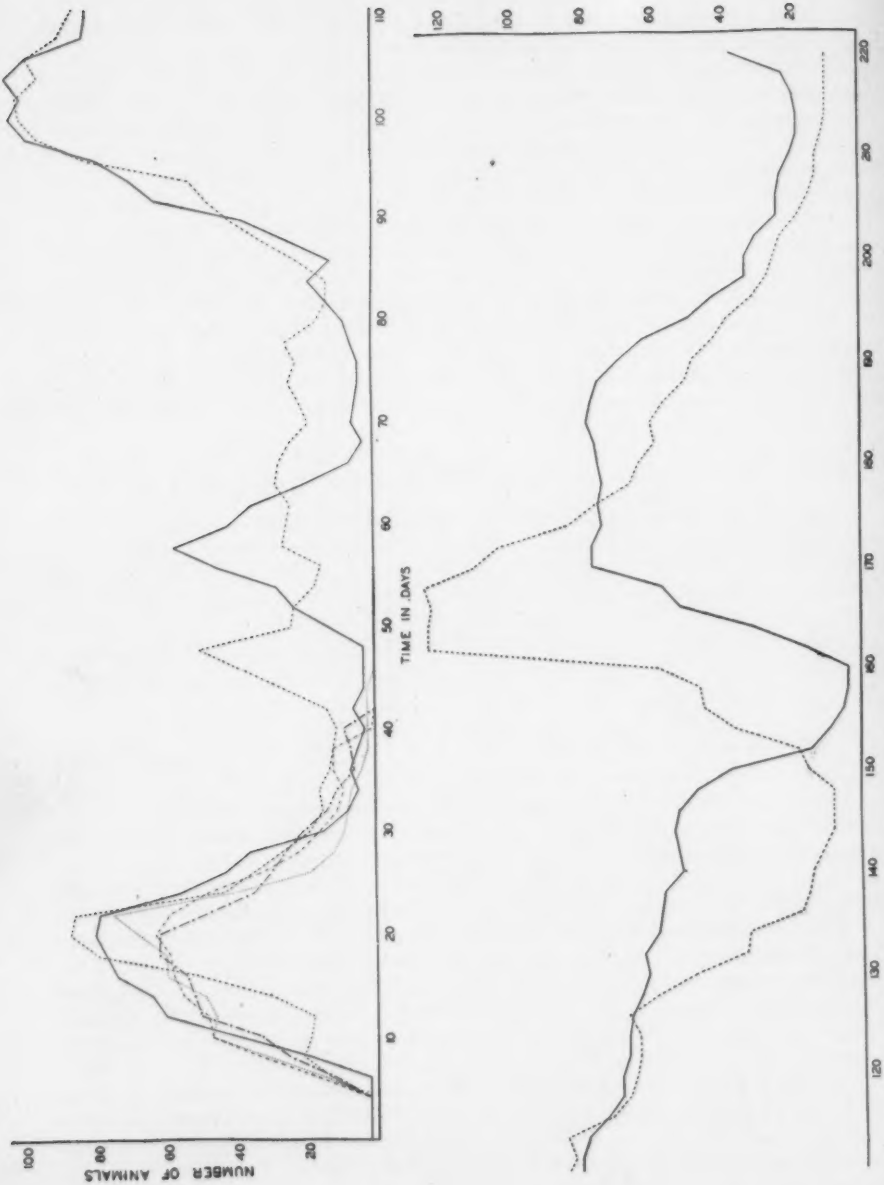
The problem of determining the influence of temperature upon population development resolves itself into two phases: 1. a descriptive study of the observable effects of temperature upon the form and dimensions of the population curve, and 2. an analysis of the processes through which the difference in temperature brings about the observed results. The present section is confined to the presentation of the factual data on the history of populations at 18° and 25° and the discussion of these growth curves. The analysis of the influence of temperature will be dealt with in later sections.

A. Observations at 25° and at 18°.

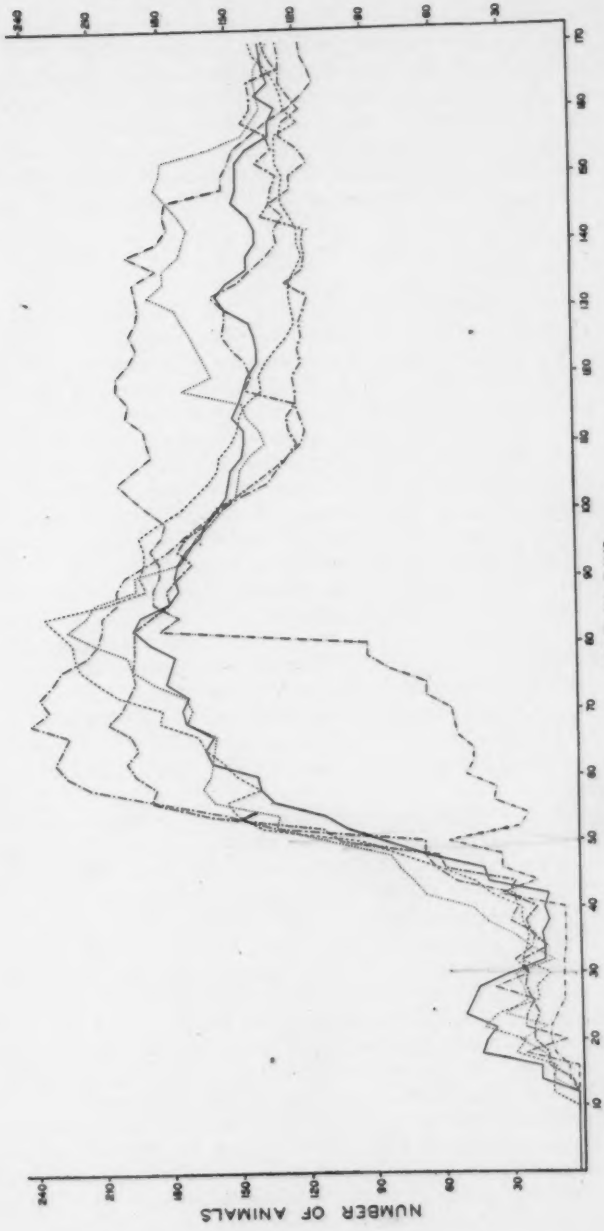
At 25°, four series of populations were started on different dates in January and February 1942. The histories of these 21 populations were recorded either until their natural extinction or until September 13, 1942, when all remaining populations were discontinued. Graph I presents the observations on a typical series, and reveals that the 25° population curve is characterized by violent and fairly regular oscillation. Instead of terminating in an upper asymptote, the first period of increase results in a pronounced peak, after which the curve drops almost to the baseline, then repeats the cycle. Typically there is no asymptote.

The majority of the populations became extinct before the experiment was terminated. Those that survived until the 234th day, when observations ceased, described, commonly, four major oscillations in numbers. The maximal size attained was a population of 126 animals.

At 18°, three series of populations, started on different dates in late March 1942, were followed until September 13, 1942, when the experiment was terminated. None of these 16 populations became extinct in the 174 days of observation. Graph II, presenting the histories of a typical series, shows that each curve described a prominent peak, followed by a gradual decrease and virtual stabiliza-



GRAPH I. Population development at 25° C. (Series C.)



GRAPH II. Population development at 18° C. (Series C.)

tion or continued oscillations of relatively minor amplitude. The greatest maximum achieved was 241 animals in the 50 cc. environment, and after the major increase, a density of more than 100 animals was permanently maintained in all the populations. While the course of development at 18° is oscillatory, it differs from that at 25° in various points that will be examined later.

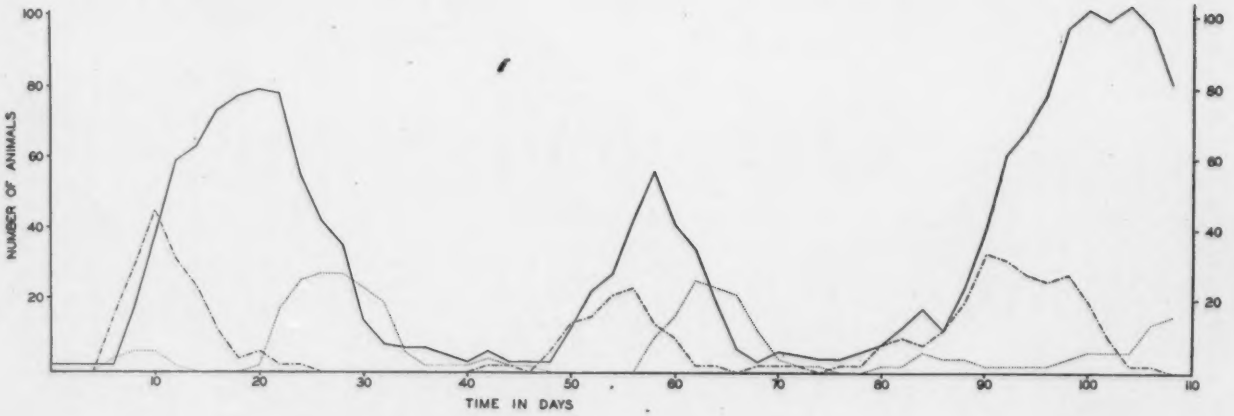
Preliminary experiments in which the volume of the medium used had been 100 cc. rather than 50 cc. yielded similar results with regard to the relative shapes of the curves, and population maxima of 381 individuals at 18° C. and 296 at 23° to 24° C.

B. Analysis of oscillation.

Since oscillation is especially pronounced at 25°, the discussion of this phenomenon will be illustrated with the data from that temperature. The analysis of fluctuations in the size of a population is essentially the study of changes in the ratio of births to deaths. Whereas the fundamental feature of an asymptotic population is that at some point birth rate and death rate become equal and thereafter remain constant, in an oscillatory population the curves describing birth rate and death rate repeatedly cross each other and never remain equal. The present fluctuations might be due to oscillation of the birth rate about a constant death rate, or to the converse, or to differential changes in both rates. In order to establish the cause of the fluctuations, it is therefore necessary to ascertain by which of these three methods the ratio of births to deaths varies.

Daily tabulation of births and deaths revealed that the oscillations observed in the 25° populations resulted from changes in both the number of births and the number of deaths. Periods of increase in population size were marked by a combination of high reproductive activity and low mortality; decreases were caused by increased mortality coupled with negligible reproduction. This mechanism of oscillation, in terms of the changing births/deaths ratio, is illustrated in Graph III which depicts part of the history of a representative 25° population (No. 2 in Graph I), with curves showing the numbers of births and deaths for each day of population census.

The history of one complete cycle will illustrate the reasons for these changes in the ratio of births to deaths. At the outset of an upward swing, the population consists in a few adults. Having lived the greater part, if not the whole, of their lives under favorable environmental conditions as regards crowding, these individuals exhibit a high reproductive rate. The growing population is composed of a few (two to 10 or 15) rapidly reproducing adults and their much smaller offspring. Graph III shows that the increasing population density begins to exert its harmful effects upon the reproductive rate before it affects the death rate, as it does in growing populations of Drosophila (Pearl, 1927). Thus the reproductive activity of the few adults in the population gradually dwindles, and the population reaches the maximum. The crucial and distinctive crossing of the birth and death curves at about this point is ascribed to two factors: 1. the extent of biological conditioning that occurs in the 48 hours between changes of the medium is presumably greater at this density than ever before, and 2. the cumulative adverse effects of crowding upon animals that have lived the greater part of their lives at high population densities begin to manifest themselves. The effect of these factors is sudden and severe: the death rate soars and reproduction is greatly



GRAPH III. Mechanism of oscillation at 25° C.

Legend: population size ————————
deaths * .....
births * -.-.-.-.-.-

* If the actual number of deaths and births occurring on each day is plotted, the resulting curves are too irregular and too low to read with ease. Accordingly, each number was doubled, and the curves smoothed by plotting the points as 3-point moving averages.

reduced. The rapidly growing population has "overshot" the density which it might theoretically be just capable of sustaining.

That the population overshoots the density of potential equilibrium does not explain why it drops dangerously close to extinction before recovering itself. At a point, for example, halfway in its descent it might be expected to rally its forces and start up again, since this same intermediate density on the upswing had been correlated with a high reproductive rate and low mortality.

In seeking the reason for the difference in performance of the two populations of equal density, it should be borne in mind that the composition of the waning population at any given point differs significantly from that of the waxing population of the same numerical strength. For example, the average size of the individuals, and hence the total biomass, of the waning population is much the greater. This fact suggests the possibility that the total metabolism of the declining population is higher than that of the growing population. If this is true, the conditioning of the medium by the accumulation of metabolic wastes or depletion of the dissolved oxygen would proceed at a greater rate in the water of the old population than in that of the younger one, and the given numerical density would exert a more severe effect upon the former than upon the latter. This might account for the difference in the subsequent histories of the two populations.

The question of comparative total metabolism was tested by determining the relative rates of depletion of dissolved oxygen in the medium. Fifty adult Daphnia chosen at random from a large culture were placed in each of six bottles with 50 cc. of pond water containing Chlorella. A similar series was made up using smaller animals from the same culture, 50 to the bottle. At the end of two days the dissolved oxygen content of the water was determined by the Winkler method, three bottles being required for each determination. The animals were then transferred to fresh pond water, 50 cc. to the bottle, for a second two-day period, after which the oxygen concentrations were again determined. The average depletion of dissolved oxygen per two-day period of conditioning was 1.19 p.p.m. in the water occupied by the 50 adults, 2.16 p.p.m. in the medium of the 50 young. In so far as the rate of oxygen depletion is a measure of metabolism, the difference between these two figures indicates that the waning population of larger biomass has a lesser, rather than a greater, total metabolism than the waxing population of equal numerical size. Thus the continued decrease of the old population cannot be assigned to a higher rate of conditioning of the medium.

There are two explanations for the continued decrease, the first of which is to be found in changes in the age structure of the population. Since the individuals of the declining population are of a greater average age than those of the increasing population, their life expectancy is of course less. Thus the difference in constitution of the waning population provides a reason for the higher daily number of deaths in this phase of the cycle.

The second reason for the continued decrease in size of the population at densities that formerly permitted increase is disclosed in the study of its previous history as compared with that of the waxing population. The components of the young, growing population had lived all of their lives, up to any density selected for comparison, at population pressures lower than the given density. They had never suffered severe crowding. But the individuals in the waning population of

identical numerical strength have lived perhaps their entire lives at densities greater than the present density.

A special experiment demonstrated that the life-long crowding experienced by the latter individuals exerts a permanent adverse effect upon their reproductive capacities. Animals were raised in a crowded condition until their first clutches were laid in the brood chamber. They were then segregated, one animal to a bottle, and their subsequent reproductive rates were compared with those of animals reared in isolation. Unfortunately this early experiment was conducted under conditions slightly different from those obtaining in the present population studies: the volume of water used was 100 cc. rather than 50; and the temperature, not controlled, varied between 22° and 27° C. The "crowded" state was a population density of 25 animals/100 cc. Animals that lived under these conditions as young (i.e. until the sixth day) and then were segregated, each into 100 cc., exhibited a reproductive rate only 62.7 per cent of that of animals that had never suffered crowding.

It should be noted that this drastic effect was brought about by crowding of only a very moderate intensity, as compared with that experienced by the declining populations under discussion. Individuals whose previous history of crowding has included population pressures ranging from 60 to 120 animals/50 cc. no doubt suffer a far more severe inhibition of reproduction. Furthermore, it is believed that subjection to high densities during early life has a lasting deleterious effect upon survival as well as reproduction, which would help to explain the persistence of a high death rate as well as a low birth rate in the shrinking population.

The effects of previous crowding may be sufficiently severe and persistent to inhibit reproduction in the waning population completely and permanently. In this event the population becomes extinct. As a rule, however, a few young are produced toward the close of the cycle, pass their juvenile stages at minimal population pressures, and attain maturity with their reproductive capacity unimpaired by crowding. From these animals stems the next growth cycle of the population.

Oscillation consists essentially in the successive "overshooting" and "undershooting" of a theoretical equilibrium density. These phenomena appear to be due to a delay, rather than a prolongation, in the manifestation of density effects. The growing population withstands a high degree of crowding with a negligible mortality. There is a lag before the effect of these population pressures is fully felt upon the death rate, which, once raised, remains high for several days while the decreasing population passes through formerly favorable densities. Likewise the reproductive rate of the increasing population is at first unaffected by high densities, but when finally checked, does not recover from the effects of crowding until long after that state of crowding has ceased to exist. Thus overshooting is occasioned by a delay in the expression of the adverse effects of high densities upon reproduction and mortality, and undershooting results from a similar lag in the manifestation of the beneficial effects of favorable densities.

C. Comparison of Oscillation at 25° and 18°.

The principal point of contrast in form of population curve at the two temperatures is the continued oscillation at 25° as compared with the tendency of the 18° curves to approach an equilibrium value.

Table I presents the duration, size-range, and mean size of the equilibria established at 18° and at 25°, and the days of the respective population histories that bounded these equilibria. After a single peak ranging from 184 to 241, oscillation at 18° was greatly reduced in all cases, six of the populations achieving nearly constant values (averaging 135.4) that they maintained until observations ceased, whereas at the higher temperature equilibria were established on only three occasions, and the general course of development was marked by a steady increase, rather than a diminution, in both the amplitude and the period of oscillation.

This progressive increase in the magnitude of oscillation at 25° is correlated with a noteworthy decrease in the rate of population extinction. Of the 21 original populations, only seven survived until the third oscillation, but of these, six were still flourishing when the experiment was discontinued. The reason for

TABLE I

Population equilibria at 18° and at 25° C.

Temperature	Population	Graph	Days bounding equilibrium	Duration	Size range	Mean size
25°	Series A, No. 3	*	30–102	72 days	8– 30	21.9
	Series A, No. 3	*	132–234	102 days	30– 54	43.4
	Series C, No. 5	I	50– 80	30 days	15– 28	22.5
18°	Series A, No. 1	*	110–174	64 days	124–145	133.6
	Series A, No. 2	*	96–174	78 days	121–157	140.7
	Series B, No. 5	*	112–172	60 days	127–156	140.4
	Series C, No. 1	II	104–170	66 days	116–144	127.6
	Series C, No. 2	II	100–170	70 days	129–157	143.3
	Series C, No. 5	II	122–170	48 days	118–140	127.0

average of mean values for equilibria at 25° C. = 29.3
average of mean values for equilibria at 18° C. = 135.4

* Graph not presented in this paper.

the improved adjustment or heightened resistance to the environment apparent in the latter half to two-thirds of population history at 25° is not clear.

Barring mutations, one cannot postulate genetic improvement through natural selection, for all the animals were genotypically identical. Since the controlled ecological conditions did not vary throughout the course of the experiment, one is led to suspect some environmental factor that was not controlled. Of these, there is only one which could conceivably have evoked the observed effect. As previously stated, no attempt was made to control conditions of light. The daylight, received from a north window, varied from season to season. It is not improbable that the amount of dissolved oxygen in the medium was an important factor in population growth. The period in which the populations appear to have been better adjusted or more resistant to their environments, beginning at the end of April, coincided with the season in which a longer daily duration of effective light enabled the food-alga Chlorella to produce a greater amount of oxygen. This added daily increment of oxygen may have been sufficient to account for the

greater success of the 25° populations in the late spring and summer months. These experiments are to be repeated, at least in part, under more rigidly controlled light conditions.

If this is the correct interpretation of the increase in amplitude and period of oscillation at 25°, evidently the 18° populations were started too late in the season to experience any such improvement in environmental conditions.

A second point of comparison is found in the nearly complete and simultaneous population "overturns" at 25°, and the more continuous overlapping of generations at 18°. At the higher temperature the first peak in numbers was due entirely to the reproduction of the seed animals, which produced several broods. Typically the first generation animals died during the first population decrease, before the appearance of the third generation. The latter individuals were not produced in numbers sufficient to prevent further decrease. None of the first or second generation animals remained at the inception of the second major increase, which was brought about by the production of the fourth generation. Thus there was a minimum of overlapping of generations. Similar population overturns, more or less complete depending upon the depth and duration of the depressions, occurred between all the subsequent peaks.

In contrast, the course of development at 18° after the major upswing was not thus punctuated by the simultaneous mass replacement of one generation by the succeeding generation. A significant feature of population history at the lower temperature was the accumulation of successive generations. The simultaneous presence of animals of all ages insured a steady replacement of adults and resulted in a sustained continuity in growth and maintenance never observed at 25°.

The lack of a sufficient number of steadily reproducing adults in an 18° population occasions the spasmodic type of population growth witnessed in the first 30 or 40 days of development. This was the period during which the second generation was being produced. The relatively infrequent production of young by the two seed animals and a comparatively high infant mortality result in a highly irregular curve. The attaining of maturity by animals of the second generation caused the tremendous increase in population size which began on about the 40th day. From this point on, the overlapping of successive generations and the constant replacement of producers gives the curve its characteristic unbroken continuity.

It should be recalled that oscillation at 25° results from an alternation of fluctuations, approximately equal in amplitude, in the number of births and the number of deaths per day. The mechanism of oscillation at 18°, in terms of the births/deaths ratio, can be analyzed in Graph IV, which is similar in purpose and in method of construction to Graph III. The data are those of population No. 1 in Graph II.

Examination of these curves reveals that there was far greater variation in the number of births per day than in the number of deaths. Moreover, the two major changes in the size of the population, viz. the tenfold increase between the 40th and 56th days, and the later more gradual decrease, were correlated, respectively, with the periods of maximal and minimal numbers of daily births. While it must be conceded that the number of deaths per day was slightly greater while the population decreased than during the period of increase, both of these levels on the deaths curve are equalled in other parts of that curve, and the difference be-

GRAPH IV. Mechanism of oscillation at 18° C.

Legend: population size ————
deaths * .....
births * - - - - -

* Plotted as in Graph III.

tween them is immaterial when compared with the variations observed in the births curve. Thus the principal changes in the size of the population are attributable to fluctuations in the number of births, while the number of deaths per day remains approximately constant. This mechanism of oscillation should be contrasted with the alternating fluctuations, approximately equal in amplitude, in the numbers of daily births and deaths that constitute the mechanism of oscillation at 25°.

The lowering of the temperature to 18° exerts a damping effect upon the magnitude and persistence of oscillation that characterizes population development at 25°. At either temperature the waxing population reaches a size that it is incapable of sustaining indefinitely. Whereas at 25° this maximum was generally less than 100 and never exceeded 126, all the 18° populations attained peaks approximately twice as high, covering the range 184–241. At 25° the effects of previous crowding upon both reproduction and longevity manifest themselves quite suddenly, and a sharp peak is described. At the lower temperature crowding acts upon reproduction alone, and its full expression is delayed longer than at 25°, with the result that the population maintains its maximum long enough to describe a short "plateau." Furthermore, whereas the 25° curve sinks almost to the baseline before increase is again possible, the waning 18° population succeeds in halting its decrease at a density which it can maintain, with minor oscillations, for at least 78 days. It should be noted that this density is greater than that achieved in any of the 25° maxima. Thus regardless of temperature, the waxing population overshoots the density of potential stabilization, but the waning population at 18° does not undershoot it as the 25° population does. This virtually terminates oscillation at the lower temperature after the first peak, in sharp contrast to the continued and progressively increased oscillation at 25°.

Influence of Temperature upon Longevity and Reproduction

To account for the observed differences in the histories of populations at 18° and 25°, a series of experiments was undertaken to determine the effect of these temperatures upon the two primary variables in population growth, namely duration of life and the reproductive rate. Since such an investigation must take into consideration the influence of population density if it is to accomplish its ultimate purpose, the experiments were so designed as to measure, at each of the temperatures tested, the mean longevity and reproductive rate of animals living at different constant densities.

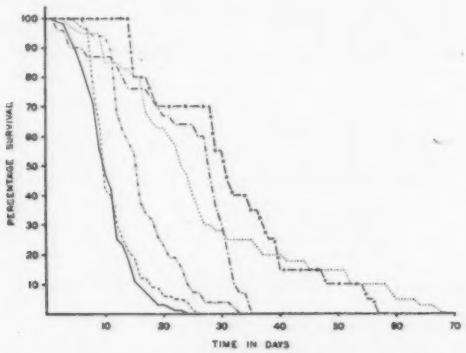
The desired number of parthenogenetically produced female Daphnia were placed in 50 cc. of fresh pond water with Chlorella added, not more than 12 hours after their release from the maternal brood pouch. The medium was renewed at two-day intervals, and the dead animals and young, when they appeared, were removed and counted daily. Population pressures of more than one animal per bottle were maintained constant by the introduction of substitute animals to take the place of those that had died. The problem of distinguishing these "substitutes" from the extant original members of the controlled population was surmounted by staining them with Neutral Red, a vital stain which in concentrations sufficient to dye the animals apparently did not injure them. (When fed only Chlorella, *Daphnia magna* does not develop the rich red color generally charac-

teristic of the species.) A staining period of 12 to 24 hours in pond water tinged with a few drops of a concentrated Neutral Red solution rendered the animals distinguishable from untreated individuals for several days. Two precautions were exercised in the selection of substitutes: 1. they were matched for size with the original members still living in the population, and 2. only individuals without eggs were chosen for this purpose. Thus, in so far as it was possible to estimate it, the substitutes' contribution to the total density effect was proportional to their number, and all of the young produced in the population were born of charter members.

A. Experiments at 25°.

Thirty tests were made at a density of one animal per 50 cc., four at densities 5 and 10, and two at densities 25, 50 and 75.

The survival curve of the 30 single animals and the average survival curves for the five higher densities are plotted on Graph V. To facilitate a quantitative



GRAPH V. Survival at different constant population densities, 25° C.

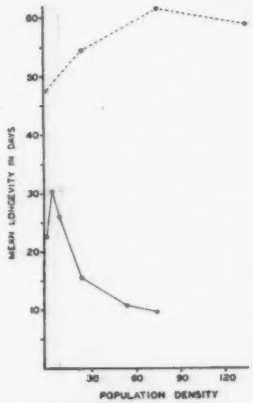
Legend: population density 1 -·-·-·-·-·-
population density 5 -o-o-o-o-o-o
population density 10 ··········
population density 25 -·-·-·-·-·-
population density 50 - - - - - - -
population density 75 ————————

comparison of survival at the six densities tested, the total number of animal days lived by each population was divided by the number of original members to give the mean duration of life at each population pressure. These data are plotted on Graph VI, which discloses the noteworthy fact that the greatest mean longevity occurs in populations of five, rather than at the minimal density, and that animals even at a density of ten per bottle lived longer, on the average, than did those in isolation.

Two interpretations of this phenomenon suggest themselves. MacArthur and Baillie (1929) have developed the thesis that the mean longevity of *Daphnia magna* is an inverse function of the metabolic rate and have reported (1929a) that

metabolic rate as indicated by the rate of heart beat is inversely proportional to population density in the range 1 to 25 animals per 100 cc. These authors did not determine the influence of population density upon longevity in Daphnia, but they implied that the reduced metabolic rate evinced by crowding might exert the same effect upon longevity as a metabolism lowered by some other process, such as decreasing the temperature. According to this theory, then, increased population pressure, up to the point of actual injury, might be expected to prolong life. MacArthur and Baillie's hypothesis may give the correct interpretation of the occurrence, observed in the present experiments, of the maximal longevity at a supraminimal density.

There is, however, a second possible explanation for this phenomenon. It was noted that the water in bottles containing only one animal was usually slightly clouded with bacteria, whereas the medium of larger populations was always kept



GRAPH VI. Population density and mean longevity.

Legend: 18° C. - - - - - - -
25° C. ———————

clear by the feeding animals. The bodies of the isolated individuals, when found dead, were frequently covered with a bacterial slime, which was occasionally observed even before death, in severe cases greatly hindering the animal's movements or even imprisoning it completely. The slime was composed of motile rods and spirilla—common fresh water saprophytes—and their gelatinous secretion. Since its appearance upon a live animal in macroscopic proportions almost invariably signalled the death of the animal within a day or two, it is believed to have contributed to the relatively high death rate at the minimal density. More crowded populations apparently never suffered from this effect; their greater numbers enabled them to maintain control of the bacterial flora.

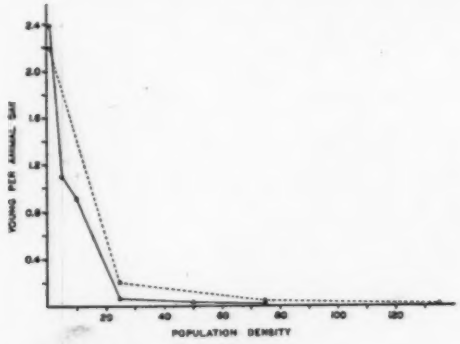
This explanation is reminiscent of one proposed to account for a similar relation between density and mean longevity observed in populations of a different animal. Allee (1931) has suggested that the positive correlation of mean longevity with population pressure in Drosophila in the density range of 1 to 35 or 55 flies per one

ounce bottle, reported by Pearl, Miner and Parker (1927) may be due to the inability of the smaller populations to keep in check the "wild" yeasts contaminating the cultures.

The available evidence does not warrant a decision between the two interpretations, metabolic and bacterial, of the results recorded here. It is not improbable that they are both operative in the present case.

The reproductive rate of each population was calculated by dividing the total number of young produced by the number of animal days lived. Graph VII, in which the results are presented, shows that reproductive rate is an inverse function of density throughout the range in which it was determined, and it drops most rapidly as the density is increased to 25 animals per bottle.

These experiments, yielding quantitative measurements of the effects of various constant densities upon longevity and reproductive rate, offered an opportunity for studying the nature of the density effect as the limiting factor in



GRAPH VII. Population density and reproductive rate.

Legend: 18° C. - - - - - - -
25° C. ————

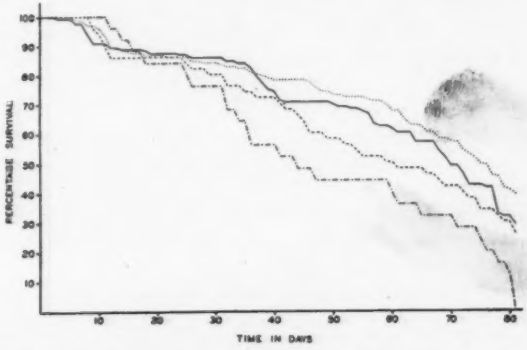
population growth. In population studies with different animals a variety of density effects have been described, but almost certainly the only influence crowding can exert upon either the birth rate or the death rate of a parthenogenetic form such as Daphnia is through the conditioning of the medium. This might lead one to expect that medium which had been heavily conditioned would exert the same adverse effects upon reproduction and longevity as those brought about by actual crowding. To test this, individual Daphnia were reared in isolation in the water conditioned by the populations of different constant densities. Every other day their medium was renewed with that which one of the larger populations had been conditioning for the past two days. Contrary to expectation the isolated animals suffered thereby no impairment of reproductive capacity. Thus the conditioning of the medium is only temporary, and probably consists in the accumulation of some volatile inhibitory substance, such as carbon dioxide, or the depletion of the dissolved oxygen supply.

Further experiments have been planned to ascertain more exactly the nature

of this limiting factor. Moreover, individual Daphnia are to be raised in medium effectively connected with that of animals living under crowded conditions, so as to test the possibility of a density effect induced directly by crowding *per se*, rather than indirectly through the conditioning of the medium.

B. Experiments at 18°.

Twenty-five tests were run at a density of one animal per 50 cc., two at densities 5 and 10, and one at density 135. On the 81st day of observation, when these experiments had to be discontinued for lack of time, all the animals at the minimal density were dead, but some individuals were still living at each of the three higher densities. The data are therefore complete for density 1 but must be regarded as partial only for densities 25, 75, and 135. Had the experiments gone to completion, the mean longevities for the latter three densities obviously would



GRAPH VIII. Survival at different constant population densities, 18° C.

Legend: population density 1 -·-·-·-·-·-
population density 25 – – – – – –
population density 75 · · · · · · · · ·
population density 135 ————————

have been higher than those obtained, and it is impossible to say whether the average reproductive rates would have differed appreciably from those observed, and in which direction. Of the two sets of data, those concerning reproduction may perhaps be considered the more accurate.

The range of densities tested was extended to include the mean equilibrium value, 135 (see Table I, p. 126), in the 18° populations. The survival curve of the 25 single animals and the average survival curves for the other densities are plotted on Graph VIII.

Calculation of the mean longevity by dividing the total number of animal days by the density of the population yields results which, for densities 25, 75 and 135 are obviously only minimal, since some animals were still living at those densities when observations ceased. The data are shown in Graph VI.

The mean reproductive rates for the 81 days of observation are presented in Graph VII.

C. Comparison of results, 25° and 18°.

Throughout the range of population densities tested, duration of life is greater at 18° than at 25° (Graph VI). This results from the positive correlation of metabolic rate with temperature and the negative correlation of longevity with metabolic rate.

The action of population pressure is quite different at the two temperatures. Whereas a density of 5 was found to be the optimum for duration of life at 25°, throughout the density range 1 to 75 longevity at 18° is a direct function of population pressure, and animals living at density 135 lived, on the average, longer than did those in isolation. The bacterial contamination of the medium which is believed to have contributed to the death rate of single individuals at 25° was never observed in the 18° bottles. This is, then, an indirect effect of temperature upon duration of life: even at the minimal density the colder water did not support a bacterial flora sufficiently concentrated to injure the animals. The absence of a bacterial effect at 18° makes it seem likely that the positive correlation of density and longevity in this case is incidental to a lowered metabolic rate. At 18° the population pressure at which metabolism is depressed to the point of positive injury lies somewhere in the density range 25 to 135, perhaps at about 75 animals per bottle.

Whereas a decrease in temperature of 7° raised the mean longevity, presumably through depression of the metabolic rate, it did not thereby bring about an equivalent reduction in reproductive rate. Apparently the rates of reproduction and mortality are not dependent upon exactly the same physiological processes. If they were, a given increase in longevity with a reduction in temperature would be correlated with a decrease in birth rate of the same magnitude.

The action of increasing density upon reproductive rate is very similar at the two temperatures (Graph VII), although it is slightly more severe at 25°: at the minimal density birth rate at 25° is higher than at 18°, but drops faster with increased crowding and at density 25 is slightly lower than the corresponding 18° rate.

The relative potential rates of population increase (i.e. the rates that would obtain if there were no density effects) can be calculated by comparing the data for the minimal density at the two temperatures. The birth rate at 18° is 2.19 young per animal day, or a gross factor of daily increase of 2.19X. The death rate (which is the reciprocal of the mean longevity, or 1/47.6) is .021X per day. Thus the net rate of potential daily increase (2.19X–.021X) is 2.17X. At 25°, gross increase (2.38X) minus death rate (.044X) yields a net rate of potential daily increase of 2.34X. When one considers that birth rate at 18° is only slightly less than at 25° and that longevity at 18° is more than twice as great as at 25°, this result is perhaps astonishing, but it illustrates the fact that birth rate is so much greater than death rate as to be the only effective factor in the net rate of increase. The reproductive rate taken alone gives the 25° population an initial advantage of .19X (2.38X–2.19X) over the 18° population. Granting the observed 25° death rate of .044X, the net rate of potential increase at 18° could not equal that at 25° even if death rate at the lower temperature were reduced to zero. But, as we have seen, the differential action of population density is such that ultimately a population attains a greater size at 18° than at 25°.

It will be recalled that oscillation at 25° was brought about by an alternation of approximately equivalent effects of population density upon the number of births and the number of deaths. The results of the reproduction and longevity experiments at 25° bear out the contention that population density at that temperature affects both of the primary variables in population growth, in opposite directions and to approximately the same extent. On the other hand, the reproduction and longevity experiments at 18° give results consistent with the observation that the mechanism of oscillation at that temperature was the fluctuation in the number of births about a nearly constant daily number of deaths. Population pressure has a relatively insignificant effect upon mortality. Moreover, if the number of deaths per day in an increasing population remains constant, the death *rate* must be an inverse function of population density. This deduction is supported by the fact that mortality in the reproduction and longevity experiments was lower at density 135 than at the minimal population pressure. This action of density upon death rate, operating in generally the same direction as the effect of density upon birth rate, tends to moderate, rather than intensify, the severity of oscillation. Therefore oscillation at 18° must be attributed wholly to changes in birth rate.

From the reproduction-longevity data one can calculate theoretical asymptotes for populations at 18° and at 25°. The number of young produced by an individual of mean longevity and reproductive rate at a given density can be determined by dividing the total number of young born at that density by the size of the population. These figures for the four population pressures tested at 18° C. are as follows:

density	1	25	75	135
average number of young/individual	104.5	10.7	0.73	0.19

Obviously a population of such density that each member could just replace itself before dying should be capable of maintaining a constant size. It is found by interpolation that the density at which the average animal produces one young in the course of its life is 73.6. It should be noted, however, that this theoretical asymptotic value is considerably lower than the mean of equilibrium values (135.4) actually established in the 18° populations. The discrepancy is serious, and perhaps cannot be entirely explained by the fact that the reproduction-longevity experiments did not go to completion.

At the higher temperature the agreement between observed equilibrium values and the theoretical asymptote is much closer. The number of young produced per individual in the 25° reproduction-longevity experiments is as follows:

density	1	5	10	25	50	75
average number of young/individual	53.9	26.7	23.1	0.82	0.26	0.08

The calculated asymptote is 24.8 animals per bottle, while the mean of equilibrium values actually observed in the 25° populations is 29.3.

The explanation for the discrepancies between observed and calculated equilibrium values is not clear. It is suggested that the age-structure of the population is a significant factor. Apparently the conditions implied by a density of a given number of animals of the same age are different from those implied by a density of the same number of animals of different ages. Although the repro-

duction-longevity experiments at both temperatures yield theoretical equilibrium values that are probably lower than the actual levels of stabilization, these experiments undoubtedly give a faithful picture of the relative effects of different densities upon the reproductive rate and upon longevity, and the data for the minimal densities can be regarded as absolute, under the given conditions.

Discussion

A. Oscillation.

Fluctuations in the density of populations in nature can usually be assigned to changes in environmental forces, which may be physico-chemical or biotic. The environmental disturbance may evoke an immediate response in the numbers of the species under consideration (as in the case of epidemics, sudden changes in meteorological conditions, etc.), or its action may be delayed for a longer interval. An example of this second category is the determination of the future size of adult populations of marine fishes by the effects of various environmental agencies upon the early developmental stages (Hjort, 1914; Johnstone, 1928). Presumably the periodic oscillations in the numbers of fur-bearing mammals and game birds which have been synchronized with sunspot cycles (Elton, 1924; Gross, 1931; Naumov, 1939; Braestrup, 1940; Green and Evans, 1940) result from complexes of environmental vectors whose action is more or less delayed.

When one considers the instability of the environment, it is not surprising that natural populations undergo violent fluctuations. However, it has been argued from mathematical grounds that the interaction of two or more animal species, e.g. predator and prey (Volterra, 1926) or parasite and host (Nicholson, 1933) is such as to give rise to rhythmic pulsations in the numbers of the animals, even though the environment is maintained constant in all other respects. In such a situation, oscillation in population density would be attributable to biotic forces exclusively, the physico-chemical factors of the environment being fixed.

Of the cases of fluctuations in numbers whose cause has been ascertained, all that have come to my attention are laid to variations in some external agency. The oscillations in Daphnia populations discussed in this paper are of an essentially different nature. Here the agent of fluctuation is internal and intrinsic. While the environment plays an important role, it is an environment whose critical changes are determined by the activities of the animals themselves. The cause of oscillation is the delay in the action of population density upon mortality and the reproductive rate, rather than a variation in some external environmental agency. It is obvious that fluctuation would not occur if the effects of a given density upon birth and death rates manifested themselves immediately; an increasing population would gradually develop an asymptote instead of "overshooting." Thus the ultimate source of oscillation is a lack of synchronization of a physiological state with the forces that provoke it.

B. Influence of temperature on population size.

It was originally intended to obtain a quantitative expression of the influence of temperature on population size by comparing the asymptotes developed at the different temperatures. Since this is clearly impossible, apparently the most satisfactory comparison would be one involving the mean sizes of the populations.

The average sizes of the 21 populations at 25° covered a range of 18.5 to 43.0, with the average at 32.6. The range of average sizes of the 16 populations at 18° was 104.7 to 126.2, the mean 112.4. Thus the mean of population size at 18° was about two and one half times as great as at 25°.

This result is consistent with the common experience that populations, and particularly those of marine plankton, attain greater densities in cold than in warmer regions (Oltmanns, 1923; Bělehrádek, 1935; Welch, 1935; Russell and Younge, 1936; Hesse, Allee and Schmidt, 1937). It should be borne in mind that this greater abundance in polar waters refers to the size of the equilibrium population, rather than to the productivity in terms of the rate of turnover.

Inasmuch as the cause of this relative abundance is still not certain, and a variety of theories have been developed to account for it, the possibility of applying the results of the present study to the problem should be of considerable interest. At the outset, however, it is apparent that the type of environmental factor preventing unlimited growth in these experimental populations (i.e. a biological conditioning of the medium by the accumulation of metabolites and/or depletion of the oxygen supply) is probably never an effective limiting factor in the open ocean. In recent years, however, it has been contended that the latitudinal variation in plankton abundance so often observed in the sea obtains in fresh waters as well (Welch, 1935), and it is quite possible that the limiting factor in the Daphnia populations is operative in some fresh water situations.

Of the various hypotheses advanced by the oceanographers, there is but one which might be applicable to the present case. This is the theory that attempts to explain the greater asymptotic level of polar planktonic populations by a direct effect of temperature upon the metabolic rate. It is argued that the lower metabolism in cold waters results in a longer duration of life and thus in an accumulation of generations; and further, that this increase in longevity more than offsets the concomitant reduction in reproductive rate. In short, the decrease in temperature exerts a greater effect on duration of life than upon the birth rate. Loeb (1912) supported this argument with the observation that the prolongation of life of sea urchin eggs with a drop in temperature greatly exceeded the retardation of their development. The theory involves only the *direct* effects of temperature upon birth and death rates. It alleges to explain the observed results without reference to any action of population density upon reproduction and duration of life.

A critical examination of the Loebian theory discloses that it really cannot account for differences in asymptotic levels. The disproportionately greater longevity at the lower temperature cannot possibly influence the height of the asymptote, since birth and death rates in a population that has attained an asymptote are equal. The equilibrium level is determined by two factors: the previous rate of increase of the population and the duration of that increase. The birth rate/death rate ratio determines the rate of population increase. It is in this ratio that the disproportionately great longevity at the lower temperature would express itself, yielding a greater net rate of population increase in colder than in warmer waters. But the second factor, namely the duration of population increase, is in no way affected by the birth rate/death rate ratio. It is determined by some limiting factor in population growth other than temperature. This limiting factor may, in turn, be influenced by temperature, but it is essentially a

result of population density. Without it, that is with no limit to the duration of increase, the population would continue growing, geometrically and indefinitely. Since Loeb's theory involves only the rate of increase and disregards the factor of duration of increase it makes no provision for any check in population growth. The inescapable conclusion is an everlasting logarithmic increase. From a slightly different point of attack this criticism may be rephrased thus. Since there are two factors involved in the asymptotic level ultimately attained, a population with an infinitesimally low rate of increase may eventually reach a greater asymptote than that developed in shorter time by another population with a much higher rate of increase. As an example, in the present experiments the potential rate of increase at 25° was found to exceed that at 18°, yet populations at the latter temperature attained the greater mean size. It should be pointed out, however, that owing to seasonal phenomena, the time element may be critical in the development of some populations in nature. Because of the brevity of the favorable season, these populations may never reach their potential asymptotes. In this case the rate of increase is the all-important factor in the size of the population at any given moment.

The explanation for the greater mean size of the 18° populations would appear to be a differential action of density at the two temperatures. The reproduction-longevity experiments revealed that increasing population pressure exerts a more severe effect upon the birth and death rates at 25° than at 18°. Possibly this result is related to the difference in solubility of atmospheric oxygen in the medium at different temperatures, but whatever the nature of the conditioning may be, the influence of temperature upon mean population size is indirect. It operates through the differential effects of population pressure. Thus the difference in temperature exerts its observed influence upon the mean of population size only by modifying the action of population density.

Summary

1. The development of populations of *Daphnia magna* was followed at two different constant temperatures. Sixteen populations were maintained at 18° and 21 at 25° C. The 50 cc. of pond water which served as medium were renewed every other day and always contained an excess quantity of the food-alga Chlorella.

2. Population development at 25° proved oscillatory in nature, four peaks occurring in 234 days, with a maximum population size of 126 animals. In the 174 days of observation at 18°, one major peak was observed (maximum 241) followed by a decrease and virtual stabilization at a population density of about 135.

3. Analysis of the oscillation disclosed that it is due to a delay in the expression of the effects of population density upon birth and death rates.

4. The mechanism of oscillation at 25° is an alternation of fluctuations in numbers of births and numbers of deaths. The mechanism at 18° is the fluctuation in the number of births about a nearly constant number of deaths.

5. Experiments with a series of population densities artificially maintained constant showed that birth rate at 25° is an inverse function of population density. At 18° the effect of density is similar but less severe.

6. Under these conditions of constant density, mortality at 25° is in general a function of population density, although the minimal mortality occurs at a

density of 5. At 18° mortality is but little affected by conditions of density, and is apparently least at about 75 animals/50 cc.

7. The mean of population size at 18° was two and one half times as great as that at 25°.

8. This fact is compared to the supposed greater density of planktonic populations in polar than in tropical waters. The results of this study cannot be applied to the problem of marine plankton abundance since the limiting factor in the present case (the conditioning of the medium by the accumulation of metabolites and/or depletion of the dissolved oxygen supply) is presumably never operative in the ocean, although it may be operative in some fresh water situations.

9. The possibility of accounting for the greater mean size of the 18° populations by reference to the direct effect of temperature upon longevity is considered but rejected. A basic fallacy is pointed out in the theory which attempts to explain by such a direct effect of temperature the greater density of asymptotic populations in polar than in tropical regions.

10. It is concluded that the influence of temperature upon mean population size observed in these experiments is indirect: the temperature difference exerts its effect only by modifying the action of population density.

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RATE OF BREAKING AND SIZE OF THE "HALVES" OF THE ARBACIA PUNCTULATA EGG WHEN CENTRIFUGED IN HYPO- AND HYPERTONIC SEA WATER

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Problem

Arbacia punctulata eggs, when centrifuged in a mixture of sea water and isosmotic cane sugar solution used in the proper proportion to keep the eggs suspended during centrifugation, break quite uniformly into "halves" of definite size (E. N. Harvey, 1931; E. B. Harvey, 1932–1941). The question arises as to whether the eggs break more or less readily in hypotonic solutions than in sea water, and whether the relative size of the two "halves" remains the same, that is, whether the extra water is distributed equally in the two halves. It has been shown by Lucké (1932b, 1940) that when the eggs are broken into halves in sea water first and the halves are then placed in hypotonic sea water, both halves swell but the heavy (red) half swells a little less than the light (white) half owing to the presence of more of the osmotically inactive material (yolk granules) in this (red) half. The present problem is concerned with centrifuging the eggs *after* they have been swollen in hypotonic sea water. It has been shown (E. B. Harvey, 1941) that the rate of breaking and the relative size of the two halves varies with the amount of centrifugal force used. With a force of 10,000 × g, which I have taken as a standard force throughout my experiments, the white (centripetal) half is slightly larger than the red (centrifugal) half. With a greater force, the red half is larger while the white half is correspondingly smaller. With a smaller force, the red half is smaller than with greater forces, and the white half correspondingly larger. In the present experiments, therefore, a uniform standard force was used, 10,000 × g.

The size of the halves obtained by centrifuging the eggs in hypo- and hypertonic sea water and subsequently returning them to normal sea water was also studied, in order to determine how the normal water balance was regained.

Methods

Before centrifuging, the eggs from one female were kept for a half hour in 60 per cent, 80 per cent, 100 per cent (control) and 125 per cent sea water, a sufficient time for them to attain equilibrium with the medium. Eggs kept for six hours in the solutions showed no appreciable further change in size. The sugar solutions added to the sea water to keep the eggs suspended during centrifugation, were made up of the same tonicity as the hypo- and hypertonic sea water. The four tubes containing 60 per cent, 80 per cent, 100 per cent (control) and 125 per cent sugar-sea water solutions were all centrifuged at the same time in each experiment;

each tube contained one part of the egg suspension to three parts of the corresponding sugar solution, this being the proper proportion to keep the eggs suspended and free to break during centrifugation. The unbroken eggs in all the tubes come to lie at the same level, so that they are all subjected to the same centrifugal force which, of course, varies with the radial distance of the layer of eggs from the axis of the centrifuge, according to the equation $F = .04 \times R$ (= radius in cm.) $\times$ (R.P.S.)^2. It is necessary to use the eggs from only one animal for one experiment since there is considerable variability in size, segregation of granules and ease of breaking in eggs from different females, but those from one female are remarkably constant in this respect. The eggs were centrifuged for three to six minutes at 10,000 $\times$ g, according to the ease of breaking of the particular batch of eggs, and were then placed in dishes of sea water of the corresponding tonicity. The measurements were made with an ocular micrometer and checked in several experiments with a filar micrometer; the figures are accurate to about 0.6 μ. The measurements recorded are the average of ten cells, made with an optical equipment giving a magnification of 400 times; the eggs lay free in the media in Syracuse watch glasses.

The experiments were performed many times with the same general results. The data obtained in a typical experiment are given in Table I A, B, C. The same eggs were used throughout the experiment.

Results

Rate of breaking (Table I A)

The rate of breaking into halves may be judged by the number of broken eggs in comparison with the number of whole, unbroken eggs obtained after centrifuging for a definite time with a definite force. When tubes containing suspensions of eggs in a 60 per cent sea water-sugar medium, 80 per cent, 100 per cent (control) and 125 per cent were centrifuged at the same time, usually for four minutes at 10,000 $\times$ g, the degree of breaking increased in the order named. In most of the experiments, practically all the eggs were broken in the 125 per cent medium while very few were broken in the 60 per cent medium. An average experiment (Table I A), gave 10 per cent of the eggs broken in the 60 per cent medium, 20 per cent in the 80 per cent medium, 70 per cent in normal sea water and practically all in the 125 per cent medium. In an experiment where only 50 per cent of the eggs were broken in the 125 per cent medium, none were broken in the 60 per cent medium. The eggs break, therefore, less readily in hypotonic sea water, and more readily in hypertonic sea water, than they do in normal sea water.

Size of the halves (Table I A; Photographs, Plate I)

When eggs are swollen in hypotonic sea water or shrunken in hypertonic sea water and then centrifuged, the increase and decrease in size is almost entirely in the white halves, the red halves being nearly the same size as those centrifuged in

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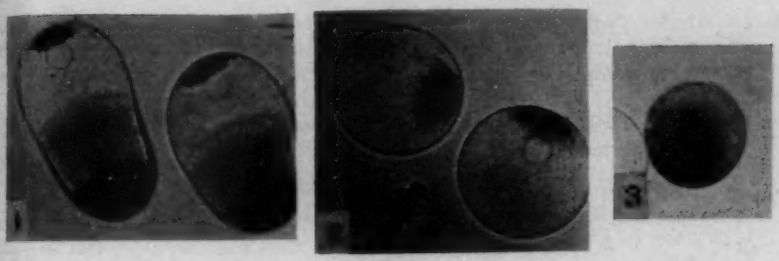
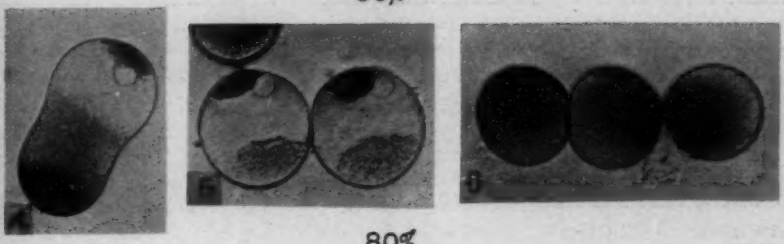
Plate I

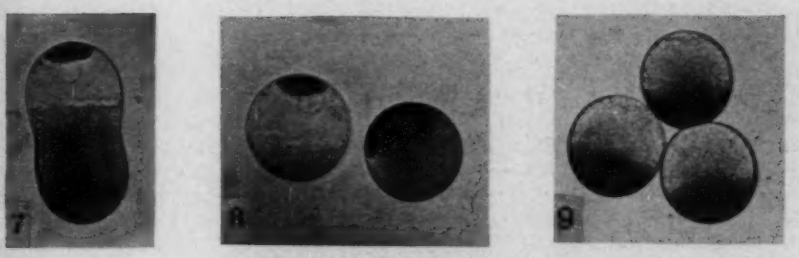
Photographs of living *Arbacia punctulata* eggs centrifuged in hypo- and hypertonic sea water, and the controls in normal sea water, and the halves into which they break with a force of 10,000 $\times$ g for four minutes. Magnification approximately 275 $\times$, all magnified exactly the same.



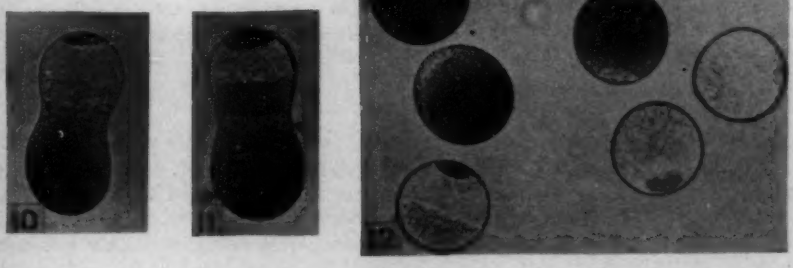
60%



80%



Control 100%



125%

PLATE I

normal sea water (Table I A). When the eggs are centrifuged in 60 per cent sea water, the white halves are very much larger than those obtained in the control (100 per cent sea water), whereas the red halves are only slightly larger than in the control (Cf. Photographs 2, 3 with 8, 9). When centrifuged in 80 per cent sea water, the white halves are somewhat larger than in the control, the red halves almost the same size (Photographs 5, 6). When centrifuged in hypertonic sea

TABLE I

Sea water	Whole egg Diam. μ	Whole egg Vol. μ³	White half Diam. μ	White half Vol. μ³	Red half Diam. μ	Red half Vol. μ³	Nucleus Diam. μ	Nucleus Vol. μ³	Per cent broken
A. *Eggs in hypo- and hypertonic sea water, then centrifuged*									
60%	82.4	(292,900)	70.4	(182,700)	58.0	(102,200)	16.0	(2,145)	10%
80%	74.9	(220,000)	62.1	(125,400)	56.3	(93,400)	12.8	(1,098)	20%
100%	72.0	(195,400)	59.0	(107,500)	56.0	(91,950)	11.5	(796)	70%
125%	66.6	(154,700)	51.7	(72,360)	53.8	(81,540)	9.6	(382)	98%
B. *Recovery in 100 per cent sea water*									
60%–100%	72.0[1]	(195,400)	59.2	(108,600)	55.7	(90,480)	11.2	(736)	
80%–100%	72.0[1]	(195,400)	59.2	(108,600)	54.7	(86,170)	11.5	(796)	
100%	72.0	(195,400)	59.0	(107,500)	56.0	(91,950)	11.5	(796)	
125%–100%	72.0[1]	(195,400)	56.3	(93,940)	57.6	(100,060)	11.2	(736)	
C. *Eggs centrifuged in sea water, then placed in hypo-hypertonic sea water*									
100%– 60%	82.4	(292,900)	67.4	(160,300)	63.4	(133,400)			
100%– 80%	74.9	(220,000)	61.8	(123,600)	57.2	(97,990)			
100%	72.0	(195,400)	59.0	(107,500)	56.0	(91,950)			
100%–125%	66.6	(154,700)	54.4	(84,300)	51.2	(70,300)			
D. *Lucké's (1932b)*[2] *mean values for C (above)*									
100%– 60%	(84.6)	317,380	(69.5)	175,560	(63.9)	136,700			
100%– 70%	(80.6)	274,020	(66.3)	152,320	(61.7)	123,060			
100%	(72.2)	197,440	(59.2)	108,600	(55.7)	90,680			

[1] These are not actual measurements because of lack of time to measure these in the same experiment as the rest. The return to normal size is approximately perfect, as determined in other experiments and as found also by Lucké and co-workers, who publish their measurements (1931a, p. 402).

[2] Only the volumes are given by Lucké; the diameters are calculated from the volumes.

water (125 per cent) the white halves are much smaller than in the control, the red halves about the same size as the controls, sometimes larger, sometimes a little smaller; the white halves are now in most experiments smaller than the red halves (Photograph 12); in a few experiments they were the same size. In the controls, the white halves were always considerably larger than the red halves (Photographs 8, 9).

Unusual batches of eggs occur occasionally, as noted in previous papers

(1936, 1941), in which, when centrifuged in normal sea water with the standard force, the red half is very small, and the white half correspondingly very large, in the ratio of 8 : 1 by volume. When such batches of eggs are centrifuged in 80 per cent sea water, the halves are of approximately the same relative size as in normal batches, in the ratio of 4 : 3 by volume, as noted previously (1941). When the unusual batches are centrifuged in hypertonic sea water, on the other hand, the relative inequality in the two halves remains; that is, the red halves are very small.

Stratification of whole eggs, and content of halves (Photographs, Plate I)

As would be expected, the granules pack much more when the eggs are centrifuged in hypotonic media than in normal sea water (Photographs 1, 4, 7). The packing of the granules takes place to such an extent in the 60 per cent sea water that the clear layer is very large and usually the white halves (Photograph 2) are almost entirely free of granules, all of them having been thrown down into the red half, although, as stated above, this red half is not much larger than the red half obtained in normal sea water which contains none of the mitochondria and only part of the yolk. This can be beautifully demonstrated in eggs stained with the vital dye, methyl green, which selectively stains the mitochondria. The purple-staining mitochondria are all in the red half. In the whole egg centrifuged in 60 per cent sea water, the mitochondrial layer is very thin, being spread over a greater area. The pigment granules are so well packed in the hypotonic solutions that the line of demarkation between yolk and pigment is very sharp, much more so than in eggs centrifuged in normal sea water. When the eggs are centrifuged in 60 per cent sea water, many of the white halves and also the upper portion of the whole eggs containing the clear layer burst soon after centrifugation; the red halves and red portion of the whole egg remain intact. This bursting is probably due to the thinness of membrane which presumably decreases in thickness as the area it covers increases.

When centrifuged in hypertonic sea water, the clear layer is small, the mitochondrial layer very thick, being spread over a small area, and in many cases is very well marked (Photograph 11). The white half is thus quite granular. The pigment is not well separated from the yolk, there being no clear line of demarkation. It is obvious from Photograph 11, that it is in general not accurate to speak of "well-stratified" eggs, since they may be well-stratified with respect to the mitochondria and poorly stratified with respect to the pigment and yolk. Many batches of eggs occur, in which, when centrifuged in normal sea water, the mitochondrial layer is indistinguishable while the yolk and pigment layers are well formed. In typical batches of eggs, however, the stratification in normal sea water is intermediate between that obtained in hypotonic sea water and in hypertonic sea water (Photograph 7).

Recovery in normal sea water (Table I B; Photographs, Plate II)

When whole normal eggs are swollen in hypotonic sea water or shrunken in hypertonic sea water, and are then returned to normal sea water, they return to normal size, as shown previously (for hypotonic) by Lucké and co-workers (1931a; 1932a). The same holds for centrifuged whole eggs. The two half-eggs

obtained by centrifuging an egg swollen in hypotonic sea water, shrink when returned to normal sea water, but not at all to the same extent. The white half loses a great deal of water, the red half very little (Photographs 13, 14). The two halves become of approximately the same size as the two halves obtained from a normal egg centrifuged in normal sea water (Photographs 8, 9). The loss of water from the white halves takes place exceedingly rapidly. Lucké and coworkers (1927; 1931b; 1932a) have called attention to the much more rapid shrinking than swelling in the case of whole eggs.

When whole normal eggs are shrunken in hypertonic sea water, and returned to normal sea water, they likewise regain their normal size. When eggs are centrifuged in a hypertonic solution (125 per cent), the two halves, as stated above, are of nearly equal size, the red half being in most batches a little larger than the white half (Photograph 12). When these halves are returned to normal sea water, they gain water in approximately the same amount and at the same rate, so that they both become slightly larger, but keep approximately the same size relative to each other (Photographs 15, 16, 17). The white halves never attain the size of the white halves centrifuged off in normal sea water. If the halves from the hypertonic sea water are placed in hypotonic sea water (60 per cent), they still swell approximately the same amount, the red halves being in the batch pictured a little larger than the whites (Photograph 18).

Size of nuclei in hypo- and hypertonic sea water and their recovery in sea water (Table I and Plate I)

Though not directly related to the problem under discussion, the size of the mature nucleus in hypo- and hypertonic sea water is of sufficient interest to be recorded here. The nucleus of a normal mature unfertilized Arbacia egg is difficult to measure because it is imbedded in granules. However, when the egg is centrifuged, the nucleus lies in the clear layer under the oil cap and can easily be observed and measured in both the whole egg and the white half. The increase in size in hypotonic sea water, and the decrease in hypertonic sea water is quite apparent in photographs (Plate I). The nucleus of the normal egg in sea water measures approximately 11.5 μ in diameter; in 60 per cent sea water the diameter is 16 μ, an increase to two and a half times its volume; in 80 per cent sea water the diameter is 12.8 μ; in 125 per cent sea water the diameter is 9.6 μ, a decrease to about one half the volume of the normal nucleus (Table I A). The cell increases to about one and one half its volume in 60 per cent, and decreases to three quarters its volume in 125 per cent sea water. The percentage increase and decrease in volume of the nucleus is greater than the percentage increase and decrease in

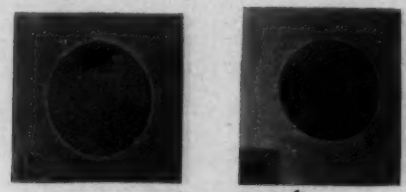
PLATE II

Photographs 13, 14. The two half-eggs obtained from centrifuging in hypotonic sea water (60 per cent, as shown in Photographs 2, 3) after their return to normal sea water.

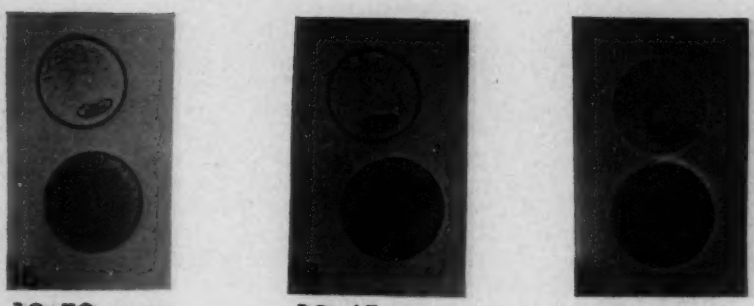
Photographs 15, 16, 17. The two half-eggs obtained from centrifuging in hypertonic sea water (125 per cent, as shown in Photograph 12) after their return to normal sea water; the same halves at 15-minute intervals. There was no further change in size in photographs taken several hours later.

Photograph 18. The two half-eggs obtained from centrifuging in hypertonic sea water (125 per cent, as shown in Photograph 12), after placing them in 60 per cent sea water.

Same magnification as in Plate I, approximately 275 ×.

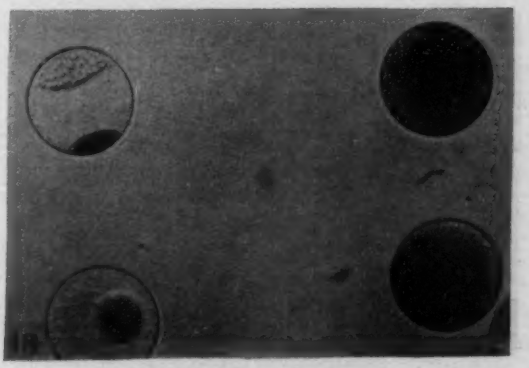


60% - 100%



12.30 p.m. 12.45 p.m. 1.p.m.

125% - 100%



125% - 60%

PLATE II

volume of the egg. This may be due to changes in metabolism, or to a smaller amount of osmotically inactive material in the nucleus, or it may be due to a difference in the nuclear and cell membranes. Skowron and Skowron (1926)[3] have noted a similar volume difference of the germinal vesicle of the *Sphaerechinus granularis* egg in comparison with the egg itself when treated with hypotonic solutions. And Beck and Shapiro (1936) found the same thing true for the germinal vesicle of the starfish egg swollen in 80 per cent sea water.

The nuclei of the whole Arbacia eggs returned to normal sea water after hypo- and hypertonic sea water, regain their normal size (Table I B), but at a much slower rate than the egg itself.

In the case of the germinal vesicle of the immature Arbacia egg, Churney (1942) concluded that it swells and shrinks reversibly in anisotonic solutions, and acts as a better osmometer than the egg itself. Beck and Shapiro (1936) have likewise found that the germinal vesicle of the starfish egg shrinks and swells in the same sense as the cell, and they have called attention to the fact that the rate is slower for the nucleus than for the egg to attain equilibrium. The mature nucleus of the Arbacia egg, therefore, seems in all respects similar to the immature nucleus (germinal vesicle) of Arbacia and other sea urchins, and of the starfish, with regard to swelling and shrinking. This is of interest and not necessarily to be expected because (1) the membrane of the mature nucleus is a new formation after the polar bodies are given off and (2) the contents of the mature and immature nucleus are different both in morphological structure (e.g. the nucleolus) and in the amount of material present; the volume of the germinal vesicle of Arbacia is about 50 times that of the mature nucleus.

Size of half-eggs obtained by centrifuging in normal sea water and then placing them in hypo- and hypertonic sea water (Table I C)

The swelling of half-eggs obtained by centrifuging eggs in a 100 per cent sea water-sugar medium and then placing them in hypotonic sea water has been adequately studied by Lucké (1932b, 1940). He found that both the half-eggs swelled in hypotonic sea water, but that the white half swelled a little more than the red half because the latter contained more of the osmotically inactive material, which he estimates as 12 per cent. His mean values are given in Table I D. My figures agree fairly well with his. In Table I C, my figures are given for the swelling and shrinking of the same eggs and half eggs as used in the other parts of the same experiment (Table I A and B). One may thus compare, in the same batch of eggs, the allocation of excess water in the two halves obtained by centrifuging before and after treating with hypotonic sea water; and similarly for the extraction of water in hypertonic sea water.

Discussion

With a constant centrifugal force of 10,000 × g, Arbacia eggs break less rapidly in hypotonic sea water and more rapidly in hypertonic sea water than they do in normal sea water. The tension at the surface is increased with the increase

[3] These authors found no decrease in size of the germinal vesicle in hypertonic glucose ($\Delta = 2.57$), though the cell shrank 57 per cent; this seems strange in view of their results for hypotonic glucose.

of surface area (Cole, 1932), so that if this factor alone were considered, the eggs should break less rapidly in hypotonic sea water, as they do. However, the densities of the half-eggs in comparison with the medium must also be considered, and these densities were not measured.

With regard to the size of the two halves, it is seen from the data presented that when Arbacia eggs are kept in hypotonic sea water and centrifuged in a similar medium, the egg breaks so that the light half is much larger than the heavy half, whereas in normal sea water it is only slightly larger. The excess water is distributed largely to the light half. Conversely, when the eggs are kept in hypertonic sea water and centrifuged in a similar medium the egg breaks so that the light half is usually slightly smaller than the heavy half. Much of the water is taken away from the light half. This is perhaps what is to be expected since it is the clear layer in the light half that contains most of the osmotically active material. Similarly, the large white halves from the hypotonic sea water lose much more water when returned to normal sea water than do the smaller, more granular red halves. On the other hand, when the eggs are centrifuged in hypertonic sea water, the granules are more evenly distributed between the two halves, now nearly equal in size. The clear layer is small, the white half is quite granular, and the granules in the heavy half are not well packed, so that there is probably more liquid (osmotically active) material present among these granules than is apparent to the eye. Thus, when these two halves are returned to normal sea water, they swell approximately the same amount.

It might be of interest to compare the results obtained with hypo- and hypertonic sea water with those previously obtained by changing the centrifugal force (1941; compare Plate I of the present paper with Plate I of the previous paper). A low force acts similarly to hypotonic sea water; the heavy granules are well segregated, the light half is much larger than the heavy half, and the egg breaks apart less readily. A high force acts similarly to hypertonic sea water; the heavy granules are not well segregated, the light half is smaller than the heavy half, and the egg breaks apart more readily. Perhaps one might conclude that when the heavy granules are well packed, whether by using a low force or by adding water, the granular half is smaller in comparison with the less granular, and the egg breaks more slowly.

Summary

1. *Arbacia punctulata* eggs, when centrifuged with a force of 10,000 × g, break less readily in hypotonic sea water, and more readily in hypertonic sea water than in normal sea water.

2. When broken apart in hypotonic sea water, the white half is very much larger than the red half. The white half is much larger than the white half obtained by centrifuging in normal sea water, the red half only slightly larger than the red half obtained in normal sea water.

3. When broken apart in hypertonic sea water, the white and red halves are of almost equal size, the white half usually a little smaller than the red half. When broken apart in normal sea water, the white half is somewhat larger than the red half. The white half from the hypertonic sea water is much smaller than the white half from normal sea water, the red half nearly the same size.

4. When the halves obtained by centrifuging in hypotonic sea water are returned to normal sea water, they both lose water, but the white half to a much greater extent than the red half. They become of approximately the same size as though they had been centrifuged in normal sea water.

5. When the halves obtained by centrifuging in hypertonic sea water are returned to normal sea water, they both take up water to about the same extent. The white half remains considerably smaller than when centrifuged in normal sea water.

6. The nucleus of the mature unfertilized egg increases perceptibly in hypotonic sea water and decreases in hypertonic sea water, to a greater percentage volume than the egg itself. It attains normal size on return to sea water.

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THE SPERMATOZOON AND FERTILIZATION MEMBRANE OF ARBACIA PUNCTULATA AS SHOWN BY THE ELECTRON MICROSCOPE [1]

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Spermatozoa have been studied for many years with the light microscope, and the general structure of many kinds of spermatozoa has been described. This study of the spermatozoa of *Arbacia punctulata* was undertaken to throw further light on their structure by the use of the electron microscope.

The fertilization membrane of the Arbacia egg which is thrown off two minutes after fertilization is now generally believed to have been, at least in part, the plasma (or cell) membrane before fertilization. The fertilization membrane was therefore studied in the hope that the electron microscope would throw some light on the structure of the plasma membrane.

Technique

The preparation of various kinds of biological material for the electron microscope has already been described in some detail (see Anderson, 1942, and references given therein). Briefly the procedure involves: (1) the complete removal of the sea water by washing several times with distilled water, in order to avoid the formation of salt crystals; (2) placing the material on a thin collodion membrane across a fine mesh wire screen (200 mesh per inch); (3) allowing it to dry; and (4) placing the screen in the electron microscope. In the present work an "RCA type B" microscope was used and the micrographs were taken with 60 kilovolt electrons.

The Arbacia sperm were taken directly from the testis of a freshly opened animal and diluted in sea water. They were then mounted on the collodion membrane to which they adhered, washed in several changes of distilled water, and then dried.

The preparation of the fertilization membranes presented greater technical difficulties; since they seemed to show no tendency to adhere to the collodion membranes, they had to be freed from the eggs and washed before they could be placed on the specimen screens. The fertilization membranes are formed about two minutes after fertilization of the eggs in sea water at 23° C. It was found that if the eggs are placed in distilled water one minute after formation of the fertilization membranes, these rupture and the egg contents flow out, leaving the empty membranes. If placed in distilled water a minute or two later, only part of the contents come out, and still later none at all. The procedure of washing

[1] We are indebted to the RCA for the use of their electron microscope at Woods Hole during the summer of 1942.

[2] Formerly RCA Fellow of the National Research Council.

the eggs several times in distilled water three minutes after fertilization was therefore adopted for separating the membranes from egg material and freeing them from salt. Under these conditions, the empty fertilization membranes sometimes retain their spherical shape, but usually collapse and become crinkled; they settle more slowly than the egg material to form a layer just above the bottom of the dish where the eggs lie. With a micropipette, under a binocular dissecting microscope, a number of membranes were taken up and deposited in tiny drops at the centers of the collodion membranes. The specimens were then dried in air and studied in the electron microscope.

Results and Discussion

Arbacia spermatozoon

The Arbacia spermatozoon at high magnification with the light microscope (Fig. 1) is observed to possess a pointed head with a flattened base adjacent to a short, slightly narrower, middle piece which seems to contain a pair of spherical bodies. The long thin filamentous tail extends from the middle piece. The head (with middle piece) measures approximately 4 μ long and 2 μ across the base; the tail is approximately 45 μ long. When placed in distilled water, the heads were observed to swell to about twice their original size.

In the electron microscope, the changes in structure caused by washing and drying are immediately apparent (Figs. 2 to 4). In most cases (except Figure 3), the heads have lost their characteristic arrow-head shape, and material appears to be flowing out of them. There is no distinct middle piece. The tails are, in most cases, coiled around the heads and consist of strands; the ends resemble frayed ends of rope unwrapped into separate strands (Fig. 2). The strands themselves are frequently detached, broken up, and strewn about the field (Fig. 3).

When examined more closely under higher magnification (Figs. 3, 4), a number of interesting features are apparent in the tails. Each tail appears to have been made up of about ten strands of uniform thickness, each having a diameter of about 50 mμ. In some of the micrographs, the tail has the appearance of a thick core surrounded by a sheath, but this appearance might be produced by a number of fibrils being superimposed at the center and flanked by one or two single fibrils. Occasionally one sees individual fibrils apparently broken up into short rods lined up in a row (Fig. 3), but this may be an artifact produced by drying, shrinking, and breaking. The regularly spaced cross striations which appear along the tail in certain areas (Fig. 4) may be characteristic of the material as has been reported for collagen fibers (Schmitt, Hall and Jakus, 1942) or may be an artifact of drying analogous to the formation of the rods noted above, but on a smaller scale.

Plate I

Figure 1. Living spermatozoa of *Arbacia punctulata* as photographed with the light microscope. × 1,000.

Figure 2. Spermatozoa of *Arbacia punctulata* micrographed with the electron microscope showing the appearance after washing in distilled water and drying. × 2,200.

Figure 3. Head and fragments of the tail of a spermatozoon at high magnification with the electron microscope. × 15,000.

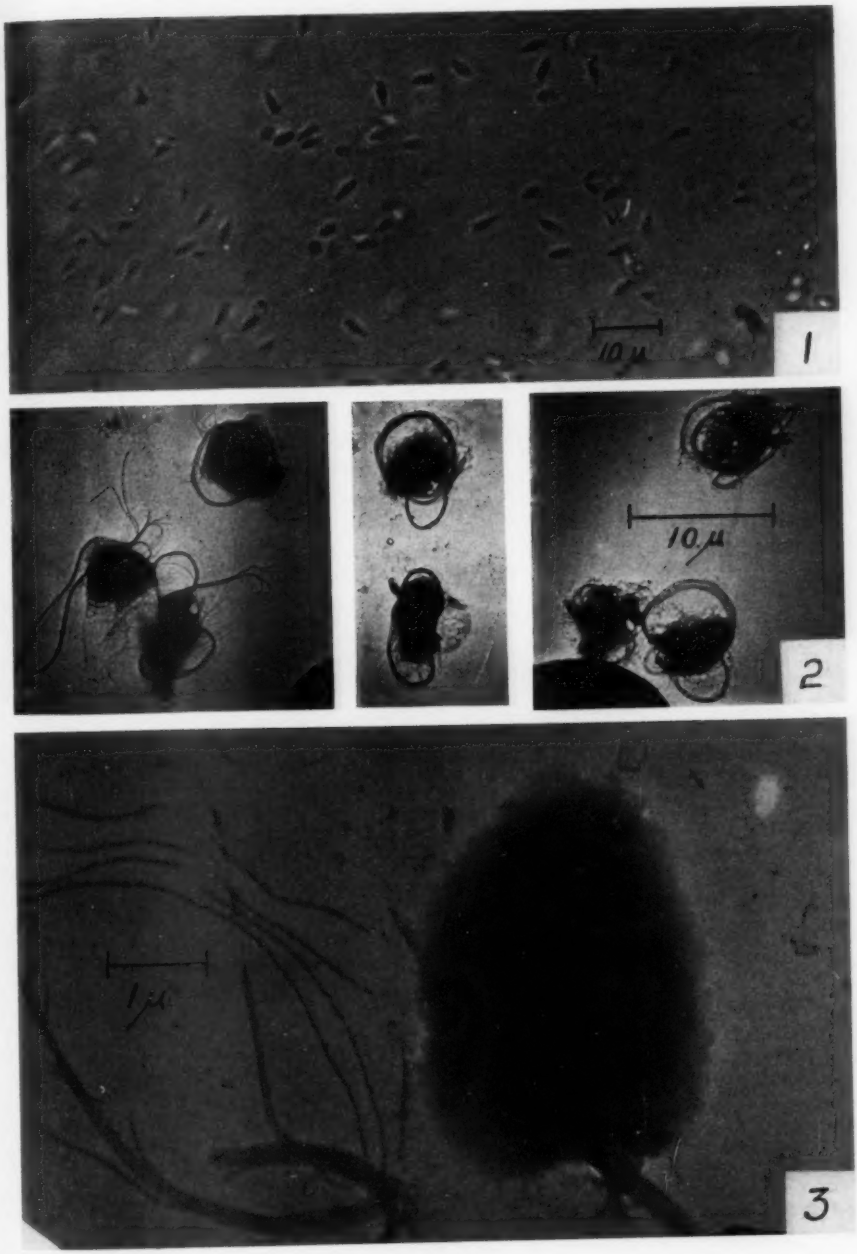

PLATE I

Unfortunately, the heads are too thick to show much internal structure. In some of the micrographs, one sees a small round area of low density which might represent a vacuole. There is also, in one of the micrographs (Fig. 4), a lighter area of the head having the appearance of a membrane. This is interpreted as the outer membrane left more or less intact on drying while the material inside has withdrawn and flowed out at the sides. It is not possible to determine the structure of the nuclear material from these micrographs. Some of the material found in the neighborhood of the heads appears to have interesting structure, such as the small rings, but it is impossible to identify it at this time.

Fertilization membrane of Arbacia

In the light microscope, the fertilization membrane of *Arbacia punctulata* appears as a uniformly thin and transparent membrane 3 to 5 μ from the surface of the egg. It is quite elastic when first formed, as shown by the fact that in high centrifugal fields it stretches from a sphere having a diameter of 80 μ to a spheroid having a length of 140 μ (Harvey, 1933, and unpublished observations). Five minutes after fertilization, however, the membrane thickens and hardens and resists stretching. Membranes freed from the eggs one minute after fertilization in distilled water have been observed to last 12 hours without any apparent change.

A number of electron micrographs of various fertilization membranes were taken and none showed anything but a thin amorphous structure (the membrane) sprinkled with what appears to be débris (Fig. 5). This débris may actually represent the structure of certain components of the cell or plasma membrane of the unfertilized egg, but the fact that they are neither characteristic in shape nor distributed in definite patterns on the surface prohibits one from attaching any special significance to them. There are no pores of sufficient size to be recognizable as such in the micrographs. From the apparent density of the micrograph one can estimate the thickness of the fertilization membrane, when first formed and dried, to be of the order of 25 mμ. It is of interest to note that this estimate is approximately the same as that of the membrane of the red blood cell. In the recent work of Zwickau (1941), who studied the red cell membranes with an electron microscope, the thickness of the membrane of the dried ghost is given as 20–30 mμ. Other estimates of the thickness of the intact red blood cell membrane, including water and diffusible proteins range from 20 mμ to as much as 50 mμ (see Ponder, 1942). The electron micrographs of the red blood cell membranes given by Zwickau show no definite structures.

PLATE II

FIGURE 4. Spermatozoon, disrupted by distilled water, showing the multiple stranded structure of the tail with cross striations, and the remains of what may have been the membrane of the head—with the electron microscope. × 10,000.

FIGURE 5. Electron micrograph of the fertilization membrane of an *Arbacia punctulata* egg. At the top of the field is the collodion film on which the specimen is mounted with a hole in it at the upper left hand corner. The fertilization membrane comes up from the bottom of the field and folds over on itself near the top. The dark line extending from the upper left hand corner is a wrinkle in the film. Note the frayed edge of the fertilization membrane to the left of the middle of this wrinkle. × 22,000.

PLATE II

Summary

1. As studied with the electron microscope, the tail of the *Arbacia punctulata* spermatozoon is found to disrupt into about ten distinct fibrils when it is washed in distilled water and dried. Each fibril is about 50 mμ in thickness. Regularly spaced cross striations also appear in the tail structure, but these may be produced in the washing and drying process.

2. A method of obtaining the fertilization membranes of *Arbacia punctulata* eggs free from egg material is described. When these were washed in distilled water and dried for examination, the electron microscope revealed no regular structures nor definite patterns. The thickness of the fertilization membrane, when first formed and dried is estimated to be of the order of 25 mμ.

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THE DEVELOPMENT OF AN ENZYME (TYROSINASE) IN THE PARTHENOGENETIC EGG OF THE GRASSHOPPER, MELANOPLUS DIFFERENTIALIS *

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INTRODUCTION

Parthenogenesis has offered opportunities for investigating problems in almost every phase of experimental biology. In the grasshopper (*Melanoplus differentialis*) parthenogenesis has been studied from the developmental and cytological aspects by King and Slifer (1934). Studies on protyrosinase formation and activation in the normal fertilized grasshopper egg have been carried out in some detail in this laboratory and it, therefore, becomes of interest to compare such results with those from parthenogenetic eggs.

MATERIALS AND METHODS

Female grasshopper nymphs (*Melanoplus differentialis*) in the third instar were segregated and raised free of males. At maturity their eggs were collected daily and kept in filter paper on moist sand at 25° C. These eggs were prepared for experimentation in the following manner: Approximately 150 eggs of a known chronological age and temperature history were placed in 0.9 per cent NaCl solution and each egg was scraped free of its chorion at the posterior end in order to determine the presence of a cuticle. Only those eggs with a cuticle were chosen. These in turn were sterilized in 70 per cent ethyl alcohol for 10 minutes. Ten to 25 eggs were taken from this lot and dissected to determine the presence of an embryo as well as its morphological age (Slifer, 1932). Of those remaining 100 were taken and placed in a glass mortar, rinsed with redistilled water, and then triturated with 0.9 per cent NaCl. The triturate was diluted to 10 ml. and centrifuged at 1,500 times G. for 10 minutes. The lipoidal *A* layer and the shell fragments constituting the major portion of the *C* layer (Bodine and Allen, 1938) were discarded since practically no protyrosinase is present in them. The protyrosinase content of the *B* layer was determined manometrically.

Each vessel of the Warburg manometer contained 1 ml. of the enzyme extract, 0.5 ml. of Sorensen's phosphate buffer (0.2 M. in respect to the phosphate) at pH 6.8, 0.3 ml. of 1 per cent aerosol OT solution, 0.9 per cent NaCl solution, and 0.3 ml. of a 0.4 per cent solution of tyramine-HCl in the side bulb. Ten minutes after equilibration at 25° C. the substrate in the side bulb was decanted into the main chamber of the vessel and the first reading taken two minutes after mixing. The manometers were shaken at 120 oscillations per minute through an amplitude of 2 cm.

* Aided by grant from the Rockefeller Foundation for research in cellular physiology.

The morphological age of the embryos in the diapause eggs was determined by removing the chorion and noting the position of the eye pigment. At diapause the head of the embryo is at the posterior end of the egg. After diapause the head of the embryo faces the anterior end of the egg making the determination of the morphological stage relatively simple (Slifer, 1932). Only those eggs which, in diapause, had a cuticle and an embryo were chosen for experiments. Such selections were especially necessary for parthenogenetic eggs to insure reproducible results.

Five day old parthenogenetic eggs were divided into two groups; one was kept as control and the other was irradiated at 1,000 r (Ray, 1938; Bodine and Allen, 1941). Both groups were then kept at 25° C. Daily determinations of the protyrosinase content were made starting with the tenth day after irradiation.

Fertilized eggs collected in the usual manner were allowed to develop at 25° C. and on the tenth day divided into two groups. One group was placed at 0° C. and the other was kept at 25° C. to serve as control. A second group of eggs was divided into two groups on the fifteenth day of prediapause development at 25° C. One group was left as a control at 25° C. while the other was placed at 0° C. A third group was divided on the fifteenth day of prediapause development at 25° C. Those placed at 0° C. were further divided after the tenth day and one lot of these was then placed at 25° C. Daily determinations of the protyrosinase content of the eggs were made.

Prediapause and diapause fertilized eggs were divided into two groups. One lot from each of these groups was kept as a control at 25° C. The other groups were separately placed into glass bottles and sealed. The eggs in the sealed bottles were then subjected for one hour to − 78° C. with the aid of dry ice and ether. The protyrosinase content of these four groups was determined immediately after the experimentals were so treated. Thereafter they were all placed at 25° C. and daily determinations of the protyrosinase content of the cold treated and control eggs were made.

Results

During prediapause from the day of laying until the fifteenth day of development at 25° C. parthenogenetic eggs contain no detectable protyrosinase (Fig. 1). Enzymogenesis begins on approximately the fifteenth day of development and the enzyme increases in amount until the twenty-fifth day. At diapause the protyrosinase content of the parthenogenetic egg remains constant at the level attained on the twenty-fifth day. Unlike parthenogenetic eggs the protyrosinase of the normal fertilized egg appears much earlier, namely on the eighth day of development (Fig. 1), (Bodine and Boell, 1935). It gradually increases in amount until at diapause the protyrosinase content is at a maximum (Bodine and Allen, 1941). It is obvious that there is a marked lag in the appearance of protyrosinase in the parthenogenetic egg. King and Slifer (1934) described a lag in the morphological, cytological and developmental aspects of the parthenogenetic grasshopper egg.

Ray (1938) observed that irradiation of normal fertilized eggs with 1,000 r on the fifth day of development destroyed the embryo but did not affect the formation of protyrosinase. Later it was noted (Bodine and Allen, 1941) that irradiation

with 1,000 r on the first day of development destroyed both the embryo and the serosa cells and that no protyrosinase was formed. Irradiation with 1,000 r on the fifth day after laying, however, was without effect on the function of the serosa cells in their formation of the yellow cuticle, white cuticle, and protyrosinase. It became of some interest, therefore, to compare the effect of a similar dose of x-irradiation on five day old parthenogenetic eggs. Since all parthenogenetic eggs do not develop (King and Slifer, 1934) it was necessary to compare the number of eggs that formed cuticle several days after irradiation with non-irradiated control parthenogenetic eggs. In both cases approximately 70 per cent formed cuticles. The presence of cuticle is important as an index of the functional state of the serosa which also seems to produce most of the protyrosinase (Bodine and Allen, 1941). In parthenogenetic eggs the formation of the cuticle begins approximately on the tenth day of development. The protyrosinase content between the fifteenth and twenty-fifth day of development was similar in irradiated and non-

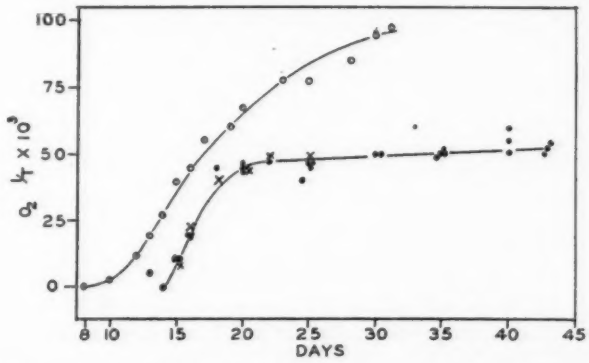

FIGURE 1. Shows amounts of enzyme in normal fertilized eggs and parthenogenetic eggs. Abscissae, developmental time in days at 25° C. Ordinate, reciprocal half oxidation period $\times 10^3$. O = normal fertilized eggs. ● = parthenogenetic eggs. X = irradiated parthenogenetic eggs.

irradiated parthenogenetic eggs (Fig. 1). It should be pointed out that in this case also the amount of protyrosinase at diapause is half as much as in the normal fertilized irradiated eggs (Ray, 1938; Bodine and Allen, 1941).

In order to check certain factors such as lag in development, high mortality, etc., possibly related to the formation of a reduced amount of enzyme in the parthenogenetic egg, the following experiments were carried out with fertilized eggs.

Fertilized eggs placed at 0° C. immediately after laying did not develop. Over a period of eighteen days no protyrosinase was detectable. When eggs so treated were placed at 25° C. protyrosinase appeared on the twenty-fifth day after laying. It should be pointed out that these eggs in reality were at a developmental temperature for 7 days $\pm$ 1 day (Fig. 2). The amount of protyrosinase and the development of the embryos were similar to control eggs kept for a similar period at developmental temperatures. Eggs placed at 0° C. on the fifteenth day of development also remained at this developmental stage. No change in the

protyrosinase content over that found on the fifteenth day was detectable. When these eggs were returned to 25° C. development and protyrosinase content increased in a normal fashion (Fig. 2). The ultimate protyrosinase content of these eggs was similar to that found in normal ones regardless of the stage or the length of time they were inhibited.

Normal fertilized diapause eggs killed by subjection to − 78° C. show a slight drop in enzyme content immediately after freezing (Bodine and Allen, 1941). Thereafter the amount of enzyme does not change significantly for a period of 18 days at 25° C. On the other hand the protyrosinase content of the normal prediapause fertilized egg remains constant for a period of 18 days after subjection to the low temperature (Fig. 3).

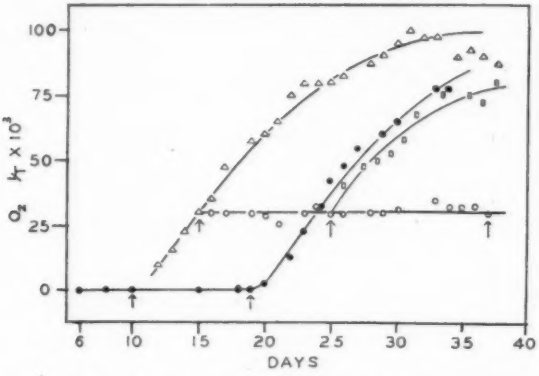

FIGURE 2. Shows effect of developmental block, due to low temperature, on the enzyme content of fertilized eggs. Ordinates as in Figure 1. Abscissae, time in days. Arrows indicate period of exposure to 0° C. △ = control eggs at 25° C. ● = put at 0° on tenth day; on eighteenth day put at 25° C. ○ = put at 0° C. on fifteenth day. □ = taken from 0° C. on the twenty-fifth day and put at 25° C.

DISCUSSION

In parthenogenetic eggs the lag in protyrosinase formation is doubtless due to the lag in the developmental aspects of these eggs (King and Slifer, 1934). The lesser amount of the protyrosinase ultimately produced in the parthenogenetic egg is a matter for some speculation.

In the course of parthenogenetic development, several anomalies occur, e.g., haploidy, retarded growth, undifferentiated growth of the embryonic cells, and a high rate of mortality (King and Slifer, 1934). Concerning a haploid condition, King and Slifer suggest that a *total* haploid condition in all probability does not permit the embryo to develop. On the other hand they have observed partial haploidy in individual embryos. They believe that in order for an embryo to develop certain of its cells essential for the propagation of the embryo, must become diploid while others may remain in a haploid condition. Haploid, diploid and triploid sets of chromosomes in individual embryos have been observed in the present work. Concerning the early differentiation of the serosa it may well be

that these nuclei in the parthenogenetic egg originate from haploid cells. Since the serosa plays such an important part in the formation of protyrosinase (Bodine and Allen, 1941) it is probable that a haploid condition may produce half the amount of protyrosinase in comparison to the normal diploid egg. We were unable to observe chromosomes in the serosa nuclei after they were morphologically differentiated. Under normal conditions the serosa nuclei in fertilized eggs increase in size and chromatin content by a peculiar type of endomitosis (Tahmisian, Allen, and Bodine, 1942). This type of growth of the serosa nuclei was also observed in the parthenogenetic eggs. As far as we can determine, normal differentiated serosa cells in general do not deviate morphologically from those found in the parthenogenetic eggs. In one case only a serosa from a parthenogenetic egg had many small nuclei interposed with normal appearing large ones.

In order to ascertain the effect of retardation in the development of normal fertilized eggs on the formation of protyrosinase, several lots of eggs were retarded

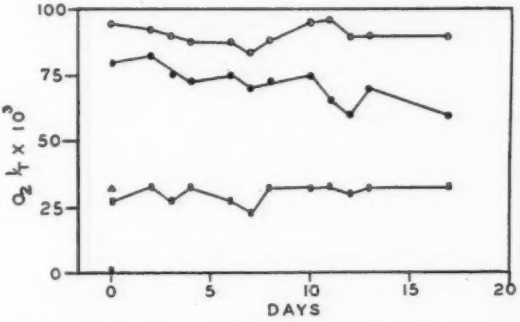

FIGURE 3. Shows effect of killing fertilized eggs by subjection to − 78° C. on their enzyme content. Ordinates and abscissae as in Figure 2. ○ = control diapause eggs. ● = diapause eggs subjected to − 78° C. △ = enzyme content of 15-day eggs. □ = 15-day eggs subjected to − 78° C.

in development by subjection to 0° C. The results of these experiments have already been mentioned above (Fig. 2). No matter at what stage the eggs were experimentally retarded in relation to the time sequence of development, the maximum amount of enzyme ultimately formed is equivalent to the amount of protyrosinase found in the control eggs. We may, therefore, conclude that the reduced amount of protyrosinase in the parthenogenetic eggs can not be accounted for on the basis that at a previous time they had been retarded in their development. Since each of the parthenogenetic eggs selected contained an embryo and a cuticle the effect of undifferentiated growth may be ruled out.

King and Slifer (1934) also pointed out that many of the parthenogenetic eggs die during development. In spite of the fact that the parthenogenetic eggs were selected each day before analysis for protyrosinase it was possible that the results were due to the presence of eggs that apparently were normal but which really were dead. In order to determine the effect of killing the eggs on their protyrosinase content, two lots killed by subjection to − 78° C. were analyzed daily. It is apparent (Fig. 3) that no detectable change in enzyme content was noted in dead

prediapause eggs for a period of 18 days. On the other hand there is a very small drop in the protyrosinase content of the killed diapause egg. As far as we can determine the parthenogenetic eggs used in all experiments were not dead. An egg, dead for 24 hours or more, changes color from a buff to dark sepia or black which in no case was observed in the parthenogenetic eggs used. We, therefore, infer that the protyrosinase content of the egg that has developed to a definite morphological age though dead contains the same amount of enzyme as a normal egg of the same age.

Another question which at present cannot be answered is suggested by the fact that all parthenogenetic eggs develop into females (King and Slifer, 1934). Is the protyrosinase content of normal fertilized female eggs one third that of the protyrosinase content of the males? If so, then a sample containing equal representation of eggs that will develop into males and females would of necessity have two times as much protyrosinase as do the eggs that will develop into females. And since all of the parthenogenetic eggs develop into females (King and Slifer, 1934), the presence of half the amount of protyrosinase in these eggs might, therefore, be accounted for on this basis.

The fact that the parthenogenetic eggs in practically all cases have exactly half as much protyrosinase as compared to the normal fertilized ones suggests that the male element donates some peculiar capacity to the normal developing egg to form twice as much protyrosinase as can be produced by the female element alone. Another possibility is that the unfertilized egg though appearing to be well coordinated for morphological differentiation is physiologically not well coordinated. It would be of interest to see if parthenogenetic eggs removed by another generation would contain still less protyrosinase.

Summary and Conclusions

1. Development and rate of growth of the enzyme tyrosinase have been studied in the parthenogenetic egg of the grasshopper, *Melanoplus differentialis*.

2. A marked lag in the appearance of the enzyme in the parthenogenetic egg occurs.

3. Total amount of enzyme found in the parthenogenetic egg is approximately 50 per cent of that found in the normal fertilized egg.

4. Parthenogenetic eggs subjected to x-irradiation on the fifth day of development show no change in the amount and rate of production of the enzyme—suggesting, as in normal eggs, the production of the enzyme by the serosa cells.

5. Results of experiments are presented which tend to show that arrested development, or killing of eggs, by low temperature do not produce lowered amounts of enzyme in eggs thus treated.

6. Possible explanations for the production by parthenogenetic eggs of lowered amounts of enzyme are given.

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A QUANTITATIVE STUDY OF ANAPHASE MOVEMENT IN THE APHID TAMALIA

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No single phase of mitosis has been discussed as often as the anaphase movement of chromosomes. The precision of the movement, the relatively large distances covered and the possibility of correlation with definite cellular structures make it better suited for causal analysis than any other phase of cell division. A great number of ingenious hypotheses have been designed to account for the movement of chromosomes, making use of practically every known chemical and physical process which could bring chromosomes from the metaphase plate to the poles. But so far none has been satisfactory and none has been verified even partly by experiment. To some extent this failure is due to the difficulty of the subject. Another reason is the over-emphasis on deductive schemes which may explain a movement of bodies like chromosomes but which are without empirical foundation. This was clearly stated by Bělař (1929a) when he pointed out that we have to find out how the chromosomes move before we can ask what forces are responsible for this movement. What is needed then is a quantitative description of the chromosome movement derived from the study of living cells in division. There are in the literature only two such accounts: one by Bělař (1929a) in spermatocytes of the grasshopper (Chorthippus) and the other by Barber (1939) in Tradescantia staminal hair cells. Bělař derived his data from measurements on photographs which were taken at intervals of several minutes. This can give only a very rough picture of the chromosome movement. Barber measured the distance between disjoining kinetochores, again on photographs, at intervals of one-half or one minute and therefore could offer a more complete description of the anaphase movement. However, the position of the long chromosomes in the metaphase plate and in early anaphase make exact measurements in these stages almost impossible. The present investigation was undertaken to provide more data on the movement of chromosomes in living cells as a basis for both experimental attacks and theoretical interpretations.

Material and Methods

The bearberry aphid *Tamalia coweni* was found to be favorable material for the study of cell division in both spermatocytes and embryonic cells. Several males or parthenogenetic females are dissected in a drop of paraffin oil on a coverglass. The testes—or young embryos—come to lie in a small pool of body fluid surrounded by paraffin oil. The coverglass is then inverted over a depression slide. Cells have thus been kept alive and normally dividing for more than 10 hours. A glass container with ferrous ammonium sulphate between lamp

and microscope prevented any heating due to the light source. The temperature varied from 22° to 26°. A good indication of the normality of conditions is given by the close agreement of the curves of different cells from different individuals (Fig. 1a and 4a). In addition spermatogonia and spermatocytes of *Protenor belfragii* and *Thelia bimaculata* were studied in a hanging drop of paraffin oil.

To analyze the movement of the chromosomes, a metaphase plate in side view is selected and with beginning anaphase the distance between the kinetochores of the daughter chromosomes recorded at intervals of one half to one minute with a camera lucida. This method was found to be simpler and more accurate than measurements on photographs. The error as determined from 20 measurements is ± 4 per cent. The various distances are then calculated in micra and plotted against time (Barber, 1939). We thus get a curve describing the movement of the chromosomes.

All forms studied here are characterized by a diffuse spindle attachment and therefore parallel disjunction. (Hughes-Schrader and Ris, 1941; Ris, 1942). This makes it easier to follow one single chromosome from metaphase to telophase. To avoid the error due to the curvature of the spindle a chromosome near the spindle axis is chosen. As a complement to the studies on live cells fixed and stained sections were used to measure the length of chromosomal fibers as well as the whole spindle with increasing separation of the daughter chromosomes.

The optics used consisted of a 2 mm. Zeiss oil immersion N.A. 1.4 and 15 × ocular.

Anaphase movement in secondary spermatocytes of Tamalia

The type of anaphase movement characteristic for the forms studied is most clearly shown in the secondary spermatocytes of Tamalia (Fig. 1a). When the daughter chromosomes begin to separate they are first connected by a "gray" mass which then breaks up into a few strands. These probably are identical with the Feulgen positive chromosome connections found in fixed cells (Ris, 1942). In a frontal or end view the chromosomes have a very characteristic dumb bell shape. The movement of the chromosomes is slow until all these connections have disappeared. Now it increases in speed and remains nearly uniform for several minutes, when it comes to a halt for about two minutes. The motion is then resumed only to slow down once more as the end of anaphase is approached. The second movement after the plateau in the curve coincides with the elongation of the cell. Previously the cell is spherical or in rare cases has elongated only slightly. Within about ten seconds after the beginning of elongation the cleavage furrow appears (arrow in Fig. 1a).

How can the interruption in the movement of the chromosomes be explained? The coincidence of cell elongation and the second movement of the chromosomes suggests that both may be connected with a stretching of the spindle. We could then picture the anaphase movement as composed of two phases: in the first the chromosomes approach the poles, or in other words, the chromosomal fibers shorten.[1] In the second phase the spindle stretches and moves the chromosomes farther apart. To prove this hypothesis we must take recourse to stained sections

[1] Since nothing is known about the mode of action of chromosomal fibers the term "shortening of chromosomal fibers" is used throughout this paper.

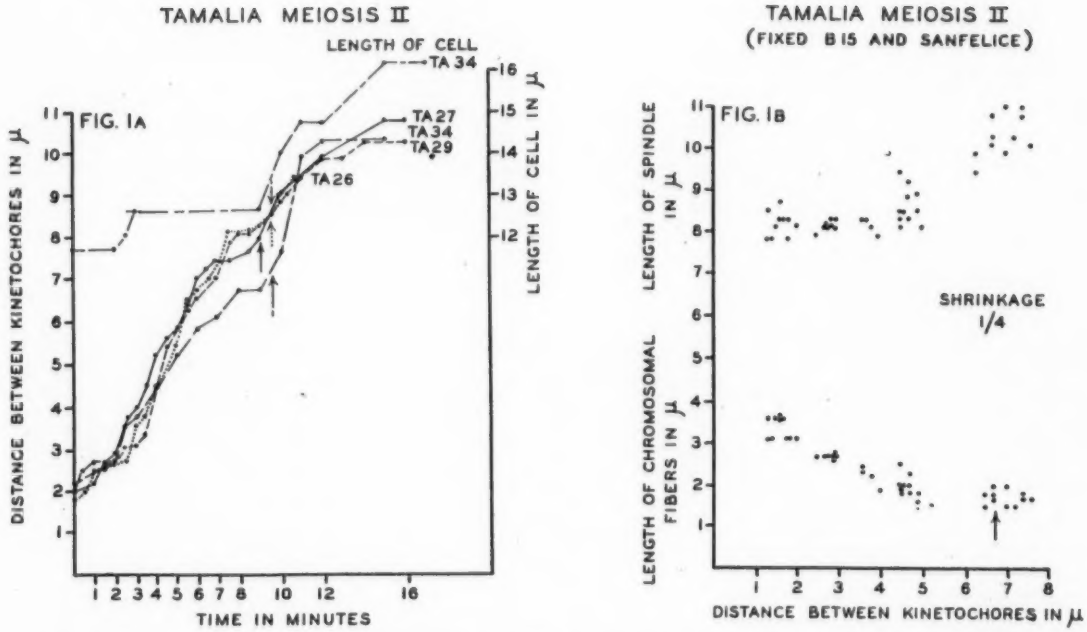


FIGURE 1a and 1b. Chromosome movement in secondary spermatocytes of Tamalia. 1a: measurements on living cells. The arrow marks the appearance of the cleavage furrow. For Ta 34 both distance between kinetochores and length of cell are plotted. 1b: measurements on fixed cells.

where we can measure the length of chromosomal fibers and spindle for various distances between the daughter chromosomes. Such measurements are plotted in Figure 1b. They show clearly that in the first part of the movement the chromosomal fibers shorten while the spindle remains constant in length. In the second phase the chromosomal fibers remain constant while the spindle begins to stretch, causing the further movement of the chromosomes. Making allowance

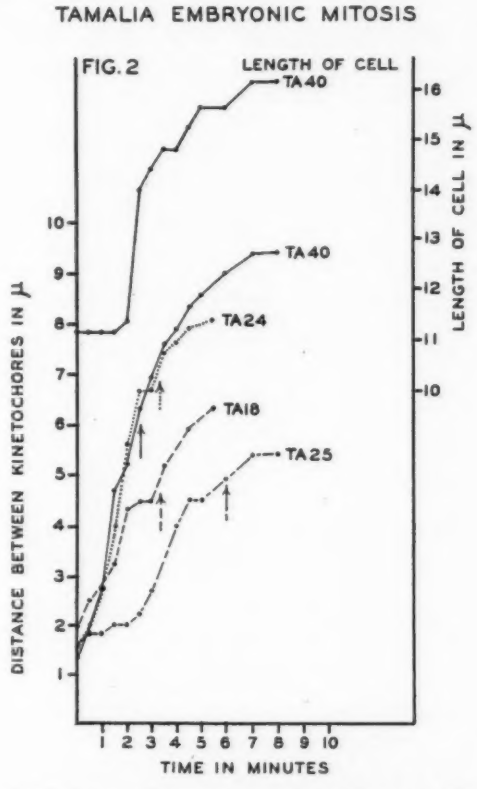


FIGURE 2. Chromosome movement in embryonic cells of Tamalia. Measurements on living cells. For Ta 40 both distance between kinetochores and length of cell are plotted.

for shrinkage at fixation, Figures 1a and 1b can be compared. Shrinkage was calculated by comparing the maximum separation of daughter chromosomes in living and fixed cells and results in a shortening of the interchromosomal distance by one-fourth. In the living cell the break in the curve occurs when the daughter chromosomes are from 7 to 8 μ apart, in the fixed cells accordingly at a separation of 5 to 6 μ. It is also interesting that the elongation of the cell corresponds closely to the increase of spindle length (increase in length of cell 4 μ, of fixed spindle 3 μ).

The movement of the chromosomes in this division can now be described in the following way: first slowly, then faster the chromosomes approach the poles apparently through the action of the chromosomal fibers. When they are from 7 to 8 μ apart this movement ceases and for a short time the chromosomes come to rest. Then the spindle begins to elongate, causing the final separation of the chromosomes. The distance from chromosomes to poles remains constant in this latter phase.

Anaphase movement in embryonic cells of Tamalia

Young embryos dissected from parthenogenetic females have many somatic cells in division. Curves for the anaphase movement are obtained as in spermatocytes. As there are many different types of cells of various sizes the curves differ quantitatively. The character of the movement, however, is the same in all cells and identical with that in secondary spermatocytes (Fig. 2). There is the initial slow movement, the first fast movement, the pause and the second movement coinciding with cell elongation. Because of the difference of

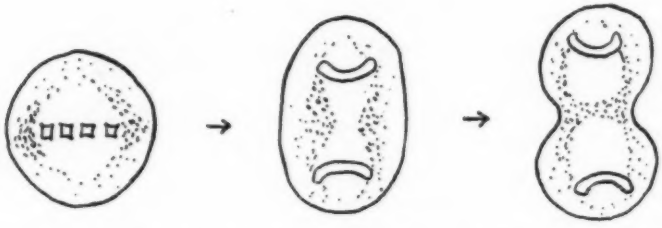


FIGURE 3. Anaphase in embryonic cell of Tamalia. Penetration of cytoplasmic granules in between the daughter plates. See text.

the cells a comparison with measurements of fixed material is impossible. Yet the curves agree well enough with those of secondary spermatocytes to justify the conclusion that the nature of the movement is the same. The velocity of the chromosomes is greater than in spermatocytes and large enough so that the chromosomes can actually be seen in motion under the microscope.

The observation of these cells during anaphase furnishes some interesting data on the spindle. The cytoplasm contains a great number of dark granules of various sizes. When the spindle is formed at metaphase they accumulate along its surface and thus outline its shape. In constant Brownian movement they can be seen bouncing off the surface of the spindle, but never penetrating it. Towards the end of metaphase the majority of granules has accumulated around the equatorial region of the spindle. In the first part of anaphase the spindle retains its characteristic shape, outlined by the cytoplasmic granules. As soon as the cell begins to elongate, indicating the stretching of the spindle, the granule-free region between the daughter plates becomes constricted in the middle and shaped like an hour glass. Soon afterwards cytoplasmic granules rush into the midregion of the spindle, continuously in unrestricted Brownian movement (Fig. 3).

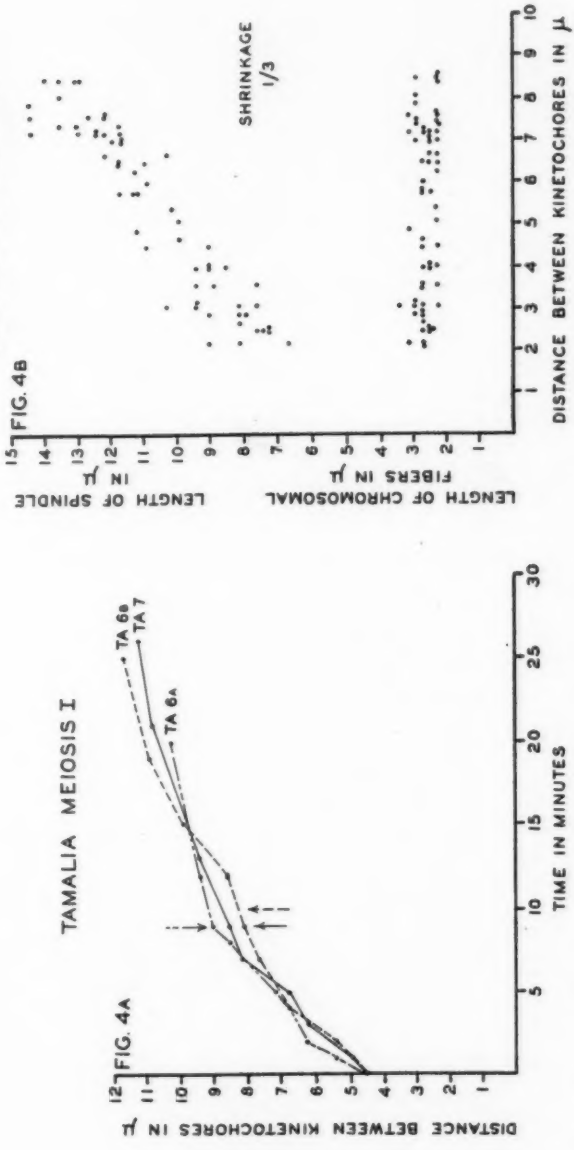


FIGURE 4a and 4b. Chromosome movement in primary spermatocytes of Tamalia. 4a: measurements on living cells. 4b: measurements on fixed cells.

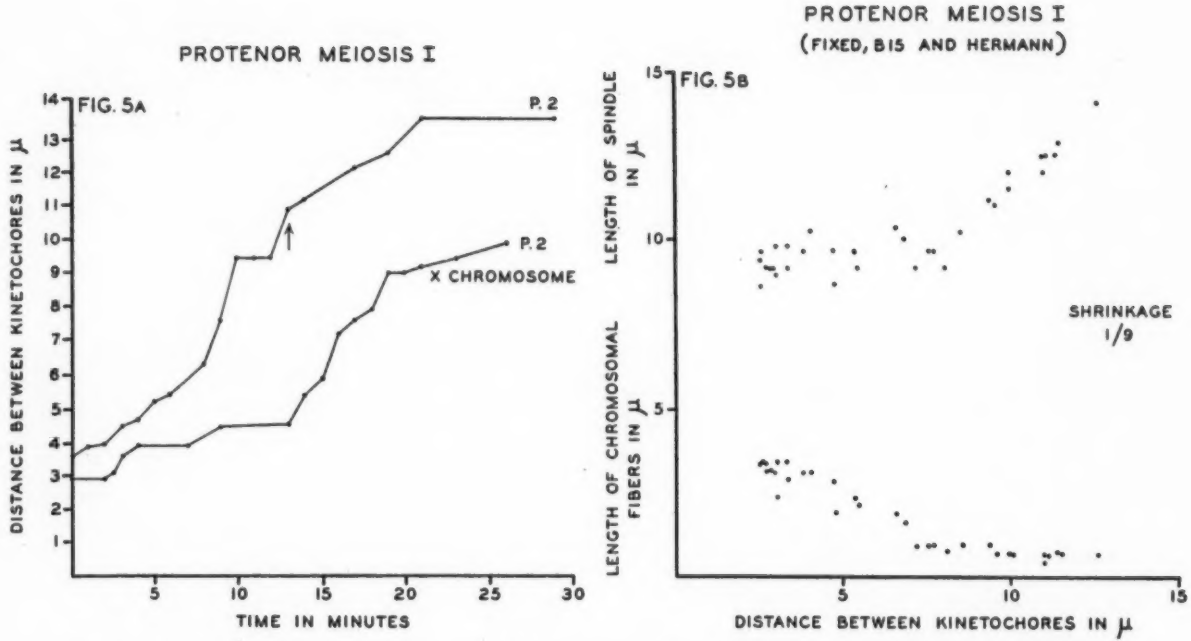


FIGURE 5a and 5b. Chromosome movement in primary spermatocytes of Protenor. 5a: measurements on a living cell, curve for autosomes and lagging X chromosome. 5b: measurements on fixed cells.

Anaphase movement in primary spermatocytes of Tamalia

The first spermatocyte division of the aphid is unusual in several ways. The univalent X chromosome is stretched into a flat sheet at anaphase and passes undivided into the larger of the unequal daughter cells (cf. Ris, 1942).

The anaphase movement also is different from that in cells previously described (Fig. 4a). The chromosomes very soon reach their maximum velocity and then gradually slow down towards the end of anaphase. The curve resembles the second movement in secondary spermatocytes, which was found to be caused by spindle elongation. Indeed the measurements of chromosomal fibers and spindle in fixed cells show that the entire movement of the chromosomes is due to the stretching of the spindle. The chromosomal fibers remain constant in length,

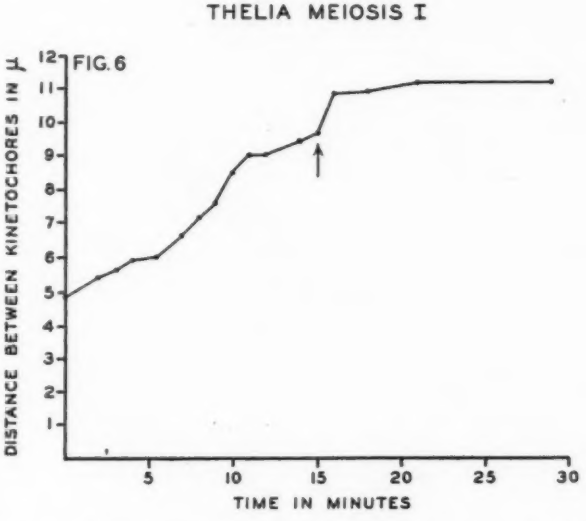


FIGURE 6. Chromosome movement in a primary spermatocyte of Thelia.

i.e., the chromosomes do not get nearer the poles (Fig. 4b). The arrow in Figure 4a marks the appearance of the cleavage furrow.

Is this kind of anaphase characteristic for primary spermatocytes or is it peculiar to the aphid? To answer this question the anaphase movement in primary spermatocytes of the hemipteran Protenor and the homopteran Thelia was analyzed.

Anaphase movement in primary spermatocytes of Protenor and Thelia

The chromosome movement in a primary spermatocyte of Protenor is shown in Figure 5a. The curve for the autosomes is of the same type as those found for somatic mitosis and secondary spermatocytes in the aphid. Again the cleavage furrow appears shortly after the second movement has started. Measurements of

fixed cells finally show that anaphase here too consists of the two phases, the approach to the poles and the spindle elongation.

Interesting is the behavior of the univalent X chromosome. In the first meiotic division it splits equationally but the daughter chromosomes lag behind the autosomes (Schrader, 1935). What is the reason for this delay? The curve for the X chromosome in Figure 5a shows that it is the first part of anaphase which differs from that of the autosomes. Chromosomal fibers are present (Schrader, 1935), but if they are responsible for the movement towards the poles, they are in some way hampered in their function. In the second phase of the movement, which is related to the stretching of the spindle, the X chromosome behaves like the autosomes and even partially catches up with them.

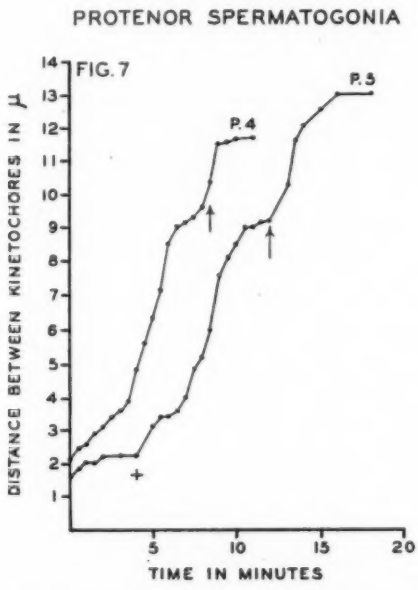


FIGURE 7. Chromosome movement in two spermatogonia of Protenor.

The first meiotic anaphase of Thelia is similar in character to that of Protenor (Fig. 6) and thus also of the same type as found in somatic cells and secondary spermatocytes of the aphid. It must be concluded, therefore, that the anaphase movement of the first meiotic division in Tamalia is different from that in Protenor and Thelia and represents an exceptional case.

Anaphase movement in spermatogonia of Protenor

In Figure 7 the anaphase movement in two spermatogonia of Protenor is recorded. In P_4 the distance between the ends, in P_5 that between the middle of two daughter chromosomes was measured. A comparison of the two curves

shows how the ends of the chromosomes separate first while the midregion lags until the daughter chromosomes are fully separated (+ in P_5). Again the movement consists of two phases, separated by a short pause.

Discussion

The measurements on living cells have furnished curves which describe in detail the movement of the chromosomes at anaphase. In the cells studied it

Table I

Anaphase movement in secondary spermatocytes and embryonic cells of Tamalia.
d.k. = *distance between kinetochores;* l.c. = *length of cell*

Time (minutes)	Spermatocytes II					Embryonic cells				
	Ta 26 25° C. d.k.(μ)	Ta 27 24° C. d.k.(μ)	Ta 29 24° C. d.k.(μ)	Ta 34 23° C.		Ta 18 24° C. d.k.(μ)	Ta 24 26° C. d.k.(μ)	Ta 25 25° C. d.k.(μ)	Ta 40 22° C.	
				d.k.(μ)	l.c.(μ)				d.k.(μ)	l.c.(μ)
0	1.8	2.0	2.0	2.2	11.7	2.0	1.6	1.6	1.4	11.2
½	2.0	—	2.5	—	—	2.5	2.0	1.8	1.8	11.2
1	2.5	2.2	2.7	—	—	2.7	2.7	1.8	2.7	11.2
1½	2.5	2.7	2.7	—	—	3.2	4.0	2.0	4.7	11.2
2	2.7	2.9	2.7	2.7	11.7	4.3	5.6	2.0	5.2	11.4
2½	2.7	3.8	3.1	3.6	12.1	4.5	6.7	2.2	6.3	14.0
3	3.6	4.0	3.1	3.8	12.6	4.5	6.7	2.7	6.9	14.4
3½	3.8	4.5	3.3	—	—	5.2	7.4	—	7.6	14.8
4	4.5	5.2	4.5	4.5	12.6	—	7.6	4.0	7.9	14.8
4½	4.9	5.6	5.4	—	—	5.9	7.9	4.5	8.3	15.3
5	5.4	5.8	—	5.2	12.6	—	—	4.5	8.6	15.7
5½	6.5	6.3	6.3	—	—	6.3	8.1	—	—	—
6	6.7	7.0	6.5	5.8	12.6			4.9	9.0	15.7
6½	7.0	7.2	—	—	—			—	—	—
7	7.4	7.4	7.0	6.1	12.6			5.4	9.4	16.2
7½	8.1	7.4	7.9	—	—			—	—	—
8	—	—	8.1	6.7	12.6			5.4	9.4	16.2
8½	8.1	7.6	8.1	—	—					
9	—	7.9	8.3	6.7	12.6					
9½	8.5	8.5	8.5	—	—					
10	9.0	9.0	8.8	7.6	14.0					
10½	9.4	—	9.0	—	—					
11	—	—	9.4	9.9	14.9					
12		9.9	9.9	10.3	14.9					
13		—	9.9	10.3	16.2					
14		—	10.3	—	—					
15		10.8	—	10.3	16.2					
16		10.8	10.3							

was found to be composed of two parts. The first can be described as the shortening of the chromosomal fibers which moves the chromosomes towards the poles. The second consists of the elongation of the spindle, resulting in a further movement of the chromosomes.

In general, this picture of anaphase agrees with Bělař's hypothesis which

resolves anaphase into (1) the action of the "Zugfaser" and (2) that of the "Stemmkörper." However, the chromosomal fibers, in the aphid at least, do not attach to a continuous fiber ("Leitfaser"), but form direct connections from the chromosome to the pole. No continuous fibers can be seen in this form. There is also little in favor of a specific differentiation of the region between the daughter-chromosomes into a "Stemmkörper." The intrusion of cytoplasmic granules into the equatorial region of the spindle (page 168) is evidence that this part of the

TABLE II

Anaphase movement in primary spermatocytes of Tamalia (Ta), *Protenor* (P), *and Thelia* (Th). d.k. = *distance between kinetochores*

Time (minutes)	Ta 6a 23° C. d.k.(μ)	Ta 6b 23° C. d.k.(μ)	Ta 7 25° d.k.(μ)	P 2 25° C.		Th 1 25° C. d.k.(μ)
				autosomes d.k.(μ)	X chromosome d.k.(μ)	
0	4.5	4.5	4.5	3.6	2.9	4.9
1	4.9	5.4	4.9	3.8	—	—
2	5.4	6.3	—	4.0	2.9	5.4
2½	—	—	—	—	3.1	—
3	—	—	6.3	4.5	3.6	5.6
4	6.7	6.7	—	4.7	3.8	5.8
5	—	7.2	6.7	5.2	—	—
5½	—	—	—	—	—	6.0
6	—	—	—	5.4	—	—
7	7.6	—	8.1	—	3.8	6.7
8	—	8.5	—	6.3	—	7.2
9	8.1	9.0	8.5	7.6	4.5	7.6
10	—	—	—	9.4	—	8.5
11	—	—	—	9.4	—	9.0
12	8.5	9.4	—	9.4	—	9.0
13	—	—	9.4	10.8	4.7	—
14	—	—	—	11.2	5.4	9.4
15	9.9	—	—	—	5.8	9.7
16	—	—	—	—	7.2	10.8
17	—	—	—	12.1	7.6	—
18	—	—	—	—	7.9	10.8
19	10.8	—	—	12.6	9.0	—
20	—	10.3	—	—	9.0	—
21	—		10.8	13.5	9.2	11.2
23	—		—	—	9.4	—
25	11.7		—	—	—	—
26			11.2	—	9.9	—
29				13.5		11.2

spindle is not a rigid "Stemmkörper," but rather less viscous than the rest of the spindle. It is more likely that the spindle as a whole elongates, though probably to a greater extent in the equatorial region. Only actual measurements can clarify this point.

The shape of the chromosomes at anaphase indicates that the chromosomal fibers exert a pull on the kinetochore. This is not only seen when the chromosomal fibers shorten and bring the chromosomes to the poles, but also in the first

spermatocyte of Tamalia where spindle elongation alone moves the chromosomes. The motion is therefore transmitted from the spindle to the chromosomes through the chromosomal fibers. The elongating spindle then does not push the chromosomes apart, but separates the poles. The chromosomal fibers, which in some way must be anchored to the polar regions then begin to pull at the spindle attachments of the chromosomes (cf. Ris, 1942; Fig. 84–90).

In the aphid, Protenor, and Thelia the two components of the anaphase movement are completely separated in time. How far can this type of movement be generalized? Barber (1939) in staminal hair cells of Tradescantia found simple S-shaped curves. He drew similar curves also through the points furnished by Bělař's photographs of anaphase in spermatocytes of the grasshopper (Chorthippus). Bělař's points are, however, so far apart that the lines drawn through them are purely hypothetical; they may or may not be simple. In

TABLE III

Anaphase movement in spermatogonia of Protenor. d.k. = *distance between kinetochores*

Time (minutes)	P 4 25° C. d.k.(μ)	P 5 25° C. d.k.(μ)	Time (minutes)	P 4 25° C. d.k.(μ)	P 5 25° C. d.k.(μ)
0	2.2	1.6	8	9.7	5.2
½	2.5	1.8	8½	10.3	6.0
1	2.7	2.0	9	11.5	7.6
1½	2.9	2.0	9½	11.5	8.1
2	3.1	2.2	10	11.7	8.5
2½	3.4	—	10½	—	9.0
3	3.6	2.2	11	11.7	9.0
3½	3.8	—	11½		9.2
4	4.9	2.2	12		9.2
4½	5.6	—	13		10.3
5	6.3	3.1	13½		11.7
5½	7.2	3.4	14		12.1
6	8.5	3.4	15		12.6
6½	9.0	3.6	16		13.0
7	9.2	4.0	18		13.0
7½	9.4	4.9			

Tradescantia staminal hair cells, as in other somatic plant cells, there is no elongation of the spindle and cell (cf. Bělař's photographs, 1929b). We may compare therefore this entire anaphase movement with the first part of that in the aphid. In both cases rather flat S-shaped curves are found. For the grasshopper preliminary measurements have shown that the chromosome movement differs from that of the aphid since the spindle begins to elongate before the shortening of the chromosomal fibers is completed.

The anaphase curve with a distinct separation of the two components is found in three Hemiptera and Homoptera, but in no other form analyzed so far. One may therefore assume that it is related to the special kind of spindle apparatus found in these forms, namely, the diffuse spindle attachment. Should this be confirmed by further studies on other forms it would give additional evidence for the functional importance of structures like chromosomal fibers still believed by

some investigators to be artifacts. It would also be an interesting example of how variations in cellular processes are related to differences in structure.

The behavior of the X chromosome in the first spermatocyte of Protenor is of great interest. Chromosomal fibers are present in metaphase and anaphase, but, as the analysis of the movement in a living cell shows, they are hindered in their normal functioning so that the X chromosome lags behind the autosomes on its way to the poles. This provides a mechanism for individual movements of chromosomes. A similar condition may be responsible for the lagging of specific chromosomes in elimination divisions of Sciara, Oligarces, etc.

The velocity of the chromosomes at anaphase is of great interest. The maximum velocities in the various divisions studied are brought together in table IV. The velocities due to the shortening of the chromosomal fibers and spindle elongation are recorded separately. The greatest velocity in embryonic cells of

TABLE IV

Maximum velocities of chromosomes. Micra/minutes. 23–26° C.

	Somatic mitosis		Spermatogonia		Meiosis I		Meiosis II	
	chromosomal fibers	spindle	chromosomal fibers	spindle	chromosomal fibers	spindle	chromosomal fibers	spindle
Tamalia	0.7–2	0.3–1.1			—	0.3	0.9–1.2	0.4–1.1
Protenor			1.3–1.6	0.3–0.5	0.9	0.7		
Thelia					0.4	0.5		
Tradescantia (Barber 1939)	1.2 (20° C.)							

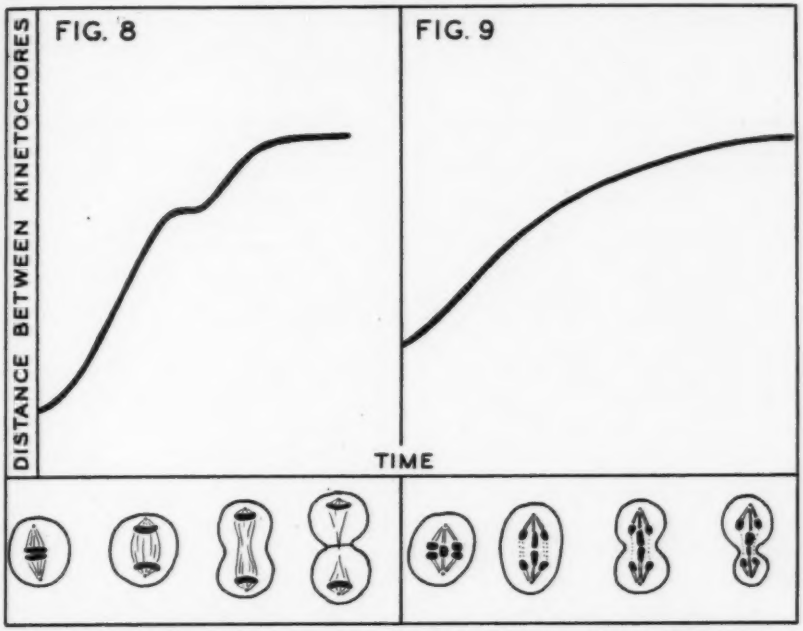
Tamalia is 2 μ per minute, or about 3 mm. in 24 hrs. As comparison the maximum velocity in Tradescantia staminal hair cells reported by Barber (1939) is added to the table.

CONCLUSIONS

The character of the chromosome movement at anaphase varies in different groups of organisms. It is possible to describe these differences as modifications in the behavior of components of the mitotic apparatus, such as chromosomal fibers and spindle body. Thus in Tradescantia staminal hair cells there is only the movement to the poles, in the first meiotic division of Tamalia only the elongation of the spindle (diagram Fig. 9). In regular divisions of Hemiptera and Homoptera the action of chromosomal fibers and spindle elongation are separated in time (diagram Fig. 8), in the grasshopper, however, they act simultaneously. These functional differences are correlated with variations in the spindle structure (diffuse against localized spindle attachment).

Measurements of chromosome movement such as those reported by Barber (1939) and in this paper represent a first step in the analysis of anaphase, namely

a quantitative description of the processes observed in the cell. The movement must then be separated into its components and related to the cellular structures which are found to be essential for regular separation of chromosomes (kinetochore, chromosomal fibers, spindle, etc.). A theory of chromosome movement must be



FIGURES 8 and 9. Diagrams illustrating the chromosome movement in forms with diffuse spindle attachment. 8: the typical anaphase curve. 9: the exceptional curve in primary spermatocytes of Tamalia.

established first on a biological plane, accounting for the many modifications of anaphase as variations of these mitotic organelles. Finally an experimental analysis of the nature of these structures and the changes they undergo during mitosis can provide an empirical basis for a physico-chemical theory of mitotic movement.

Summary

1. The movement of chromosomes at anaphase was measured in living cells of Tamalia, Protenor and Thelia. The distance between the separating chromosomes plotted against time produces curves which describe accurately the chromosome movement. In embryonic cells and secondary spermatocytes of Tamalia, spermatogonia and primary spermatocytes of Protenor, and a primary spermatocyte of Thelia the curves consist of two S-shaped components separated by a plateau. The second part of the movement coincides with the elongation of the cell.

2. In stained sections the length of chromosomal fibers and the spindle was measured at various stages of chromosome separation. A comparison with the data from living cells shows that in the first part of anaphase the chromosomal fibers shorten, i.e., the chromosomes approach the poles. In the second part the spindle elongates and thus produces a further movement of the chromosomes.

3. The chromosome movement in the otherwise exceptional anaphase of primary spermatocytes in Tamalia is characterized by a simple unbroken curve. Measurements on stained cells demonstrate that the movement is due entirely to spindle elongation. The chromosomal fibers remain constant in length and the chromosomes therefore do not approach the poles.

4. Since the double curve was found in all Hemiptera and Homoptera studied but not in the grasshopper (unpublished results) this type of anaphase movement is probably related to the diffuse spindle attachment found in these insects. This points out the functional significance of structural variations.

5. The curves for the primary spermatocyte of Protenor show that the lagging of the daughter chromosomes of the univalent X chromosome is due to an abnormal first part of the movement. This indicates some impairment in the functioning of their chromosomal fibers. The exceptional behavior of a chromosome can thus be traced to one particular factor of the anaphase movement.

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